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Michael Ryan Kovens

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**ASSESSMENT OF THREE GRAPE VARIETIES' RESISTANCE TO FIVE
VIRUSES AND ASSEMBLY OF A NOVEL GRAPEVINE VEIN CLEARING
VIRUS GENOME**

A Masters Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Plant Science

By

Michael Kovens

July 2015

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**ASSESSMENT OF THREE GRAPE VARIETIES' RESISTANCE TO FIVE
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Agriculture

Missouri State University, July 2015

Master of Plant Science

Michael Kovens

ABSTRACT

Over 65 viruses are known to infect grapevines, more than any other agricultural crop. Growth, yield, and quality of virus-infected vines suffer, reducing the profitability to grape growers. Therefore, it is critical to identify grape cultivars resistant to these viruses which can be utilized to protect more susceptible varieties from infection. Wine grape cultivar Norton (*Vitis aestivalis*) and Chambourcin (*V. vinifera* hybrid), and rootstock St. George (*V. rupestris*) have been known to be disease tolerant. In the first study, Norton and Chambourcin were infected with *Grapevine fleck virus* (GFkV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), and *Grapevine fanleaf virus* (GFLV). Norton and *V. rupestris* 'St. George' were infected with *Grapevine vein clearing virus* (GVCV). Testing of virus-infected grapevines for each virus by the polymerase chain reaction (PCR) assays indicated that the three grape varieties are susceptible to the viruses tested, and, therefore, none should be utilized as virus-resistant cultivars. In the second study, a wild *V. rupestris* 'Scheele' vine was tested positive for GVCV, which is referred to as GVCV-VRU, marking the first instance this virus has been observed in the native wild *V. rupestris* population. The whole genome (7,755bp) of the new GVCV-VRU isolate was assembled. Sequence analysis revealed that GVCV-VRU and GVCV-CHA shared 92% nucleotide identity. The discovery of a new GVCV isolate in wild *V. rupestris* grapevine and its association with vein clearing disease may have important implications for origin and management of GVCV.

KEYWORDS: grapevine, virus, resistance, aestivalis, vinifera, rupestris

This abstract is approved as to form and content

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Chairperson, Advisory Committee
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LITERATURE REVIEW

Viruses are microscopic agents capable of infecting all types of life. They are comprised only of genetic material, either DNA or RNA, surrounded by a protein coat, or capsid. Some are slightly more complex and are encased within a lipid envelope.

Viroids are less complex, containing only small circular strands of RNA molecules with no capsid. In order to cause disease, viruses must enter the host's cells and usurp host cellular pathways, such as replication, translation, and (reverse) transcription, to increase their copy numbers. In an essence, viruses are part of the host and absolutely depend on the host for survival. Thus, viruses can't be cultured *in vitro* on artificial media as most fungal and bacterial pathogens are.

Grapevines can be infected by over 65 different types of viruses as well as five viroids, more than any other agricultural crops (Martelli, 2014; Zhang et al., 2011). Commonly, a grapevine suffering from disease will be infected with a mixed population of pathogens (Komar et al., 2006; Lunden et al., 2009; Meng et al., 2006; Prosser et al., 2007; Rwahni et al., 2009). This is the cause of serious concern within vineyards worldwide. As more pathogens infect a host, the diagnosis and management of viruses becomes increasingly difficult (Rowhani et al., 2005). In turn, the healthy growth and profitability of these infected vines decrease drastically. All grapevine viruses have RNA for their genetic material except for the three DNA viruses that were recently discovered: *Grapevine vein-clearing virus* (GVCV) (Zhang et al., 2011), *Grapevine red blotch associated virus* (GRBaV) (Krenz et al., 2013), and *Grapevine Roditis leaf discoloration-associated virus* (GRLDaV) (Maliogka et al., 2015).

The report of the GVCV associated disease further complicates the matters of viral disease prevention in vineyards. The syndrome results in severe vein-clearing and vine decline, and was believed to be the result of a complex of viruses in the beginning (Lunden et al., 2009; Qiu et al., 2007), although the precise origin remains to be found. One of the most prominent symptoms is the chlorosis of both major and minor veins, giving them a translucent appearance (Qiu et al., 2007; Zhang et al., 2011). Leaves tend to have abnormal growth with crinkling and curling of the edges, and thus diseased leaves are out of normal shape (Figure 1). As the leaves mature, a mosaic pattern of green and chlorotic tissue will appear. Newly grown shoots will develop in a zig-zag pattern with stunted internodes. As the virus infection intensifies, cluster size reduces and berries become irregularly shaped and textured. Overall vigor of the vines decreases as well, resulting in less dense canopies. Eventually, death of an infected vine can occur if the infection becomes severe over time. Overall, symptoms tend to closely resemble that of nepoviruses, complicating diagnosis (Qiu et al., 2007; Zhang et al., 2011). As there is little information on the spread and treatment of GVCV, vines tend to reach the lethal point when left unattended.

GVCV belongs to the genus *Badnavirus* in the family *Caulimoviridae*. It was the first DNA virus discovered to infect grapevines. The GVCV-associated disease was first reported in 2004 on a Chardonnay vine in Missouri but GVCV wasn't identified as a novel virus until 2011. As with other members of the *Caulimoviridae* family, GVCV exists as a pararetrovirus, meaning that it must utilize an RNA intermediate. It is a non-enveloped virus comprised of circular dsDNA. The genome is 7,753 bp long and contains 3 open reading frames (ORFs) (Zhang et al., 2011). There are four short ORFs



Figure 1. Characteristic symptoms of *Grapevine vein clearing virus* (GVCV). The left panel shows the vein clearing and mosaic pattern symptoms of GVCV infection. The right panel also shows the vein clearing, as well as the backward leaf rolling.

(sORFs) within the intergenic region (Zhang et al., 2015). Viral titer has been shown to accumulate most abundantly in the petioles of young leaves (Guo et al., 2013). The only known way to transmit GVCV is through propagation of infected plant material. Insect vectors for spreading GVCV still remain elusive.

Grapevine red blotch-associated virus (GRBaV) is associated with red blotch disease and was first reported concurrently in New York and California in 2012 (Krenz et al., 2013; Sudarshana et al., 2015). Although showing homology to viruses in the genera *Begomovirus* and *Mastrevirus*, it has been placed in a new genus yet to be named, in the family *Geminiviridae* (Krenz et al., 2013). It is a non-enveloped virus comprised of a circular ssDNA that is 3,206 nt long and contains 6 bidirectional ORFs. The significance of insect vectors currently remains unknown however, the Virginia creeper leafhopper successfully transmitted GRBaV under experimental conditions. GRBaV has also been reported to be graft-transmissible. During infection of red grape varieties, red spots or blotches appear on leaves, eventually coalescing to cover most of the leaf blade. Primary, secondary, and tertiary veins, and even veinlets, become red within the blotched areas as well. Symptoms are typically less severe in white varieties, producing irregular chlorotic areas that can later turn necrotic. Also, leaves have been shown to sometimes undergo upward rolling. Ripening of the berries is delayed and the quality reduced. Because the symptoms of GRBaV are so similar to those of leafroll disease, visual diagnosis of GRBaV-infected vines becomes extremely difficult, making DNA-based assays the only method of accurate diagnosis (Sudarshana et al., 2015).

Grapevine Roditis leaf discoloration-associated virus (GRLDaV), in the genus *Badnavirus*, family *Caulimoviridae*, is associated with Roditis leaf discoloration (RLD)

disease. RLD was initially observed in the early 1980's in central Greece, primarily on the local cultivar "Roditis". However, the virus itself has only recently been identified. Like GVCV, GRLDaV is also a pararetrovirus. It is a non-enveloped virus comprised of circular dsDNA that is 6,988 bp long and contains 3 ORFs. A fourth ORF, 426 nt long, exists overlapping the last 412 nt of ORF 3. Nucleotide sequence of the RT/RNase H region in ORF 3 contains 79.7% identity with that of the same region in Fig badnavirus 1 (FBV-1), just past the 20% cut-off value used as the criterion for species differentiation in the genus *Badnavirus* according to the International Committee on Taxonomy of Viruses. GRLDaV is further separated as a species from FBV-1 by their difference in host ranges. For example, GRLDaV can readily infect *Nicotiana benthamiana*, yet FBV-1 cannot. Mealybugs, the main insect vector of most *Badnaviruses*, have yet to be examined as a possible vector for GRLDaV, but it has been shown to be able to be transmitted via mechanical means. Symptoms include yellow and/or reddish discolorations of the young leaves, as well as deformations. Yield, size, and quality of the infected grapes are all reduced (Maliogka et al., 2015).

The most widespread viruses on grapes are *Grapevine fleck virus* (GFkV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), and *Grapevine fanleaf virus* (GFLV), with the latter three also considered the most damaging (Gambino and Gribaudo, 2006; Kumar et al., 2013). GFkV, in the genus *Maculavirus*, family *Tymoviridae* (2011), infects all *Vitis* species, but is known to be symptomatic only in *V. rupestris* (Gambino and Gribaudo, 2006). It is a non-enveloped virus comprised of a linear, positive-sense ssRNA that is rich in cytosine (Martelli et al., 2002). The genome is 7,564 nt long and encodes 4 ORFs. Viral titer has been shown to accumulate the most

in the petioles of young leaves (Sabanadzovic et al., 2001) during Fall and Winter.

Transmission of GFkV is known to occur through propagation of infected plant material.

A prominent symptom of the virus is localized clearings, or “flecks,” in the veinlets of younger leaves (Figure 2). Symptoms also include wrinkling and an upward roll of older leaves, as well as a mosaic pattern. So far no direct evidence of fruit yield and quality losses associated with GFkV have been found (Constable and Rodoni, 2011).

There are nine known strains of GLRaV with strain GLRaV-3 being the most common and widespread. GLRaV-3, in the genus *Ampelovirus*, family *Closteroviridae* (2011), is associated with grapevine leafroll disease. It is the most widespread grapevine virus worldwide and is one of the most economically important viral diseases of grapevines (Tsai et al., 2011). It is a non-enveloped virus comprised of linear, positive-sense ssRNA. The genome is 184,998 nt long and contains 13 ORFs (Maree et al., 2013). Unlike GFkV, viral titer of GLRaV-3 has been shown to accumulate the most in the petioles of older leaves (Tsai et al., 2011) during Fall and Winter. GLRaV-3 has been shown to be vectored by several mealybug and soft-scale insect species and can be transmitted via propagation of infected plant material. The name of the virus comes from the downward roll of the leaves, which become thick and brittle. Discoloration can occur between major veins of leaves (Figure 2). Red varieties will encounter a reddening of their leaves while white varieties encounter a yellowing of their leaves, although, the primary veins usually remain green (Maree et al., 2013).

GVA, in the genus *Vitivirus*, family *Betaflexiviridae* (2011), is associated with rugose wood complex diseases. Some variants are closely associated with Shiraz disease (Wang et al., 2012) and some are associated with Kober stem grooving disease

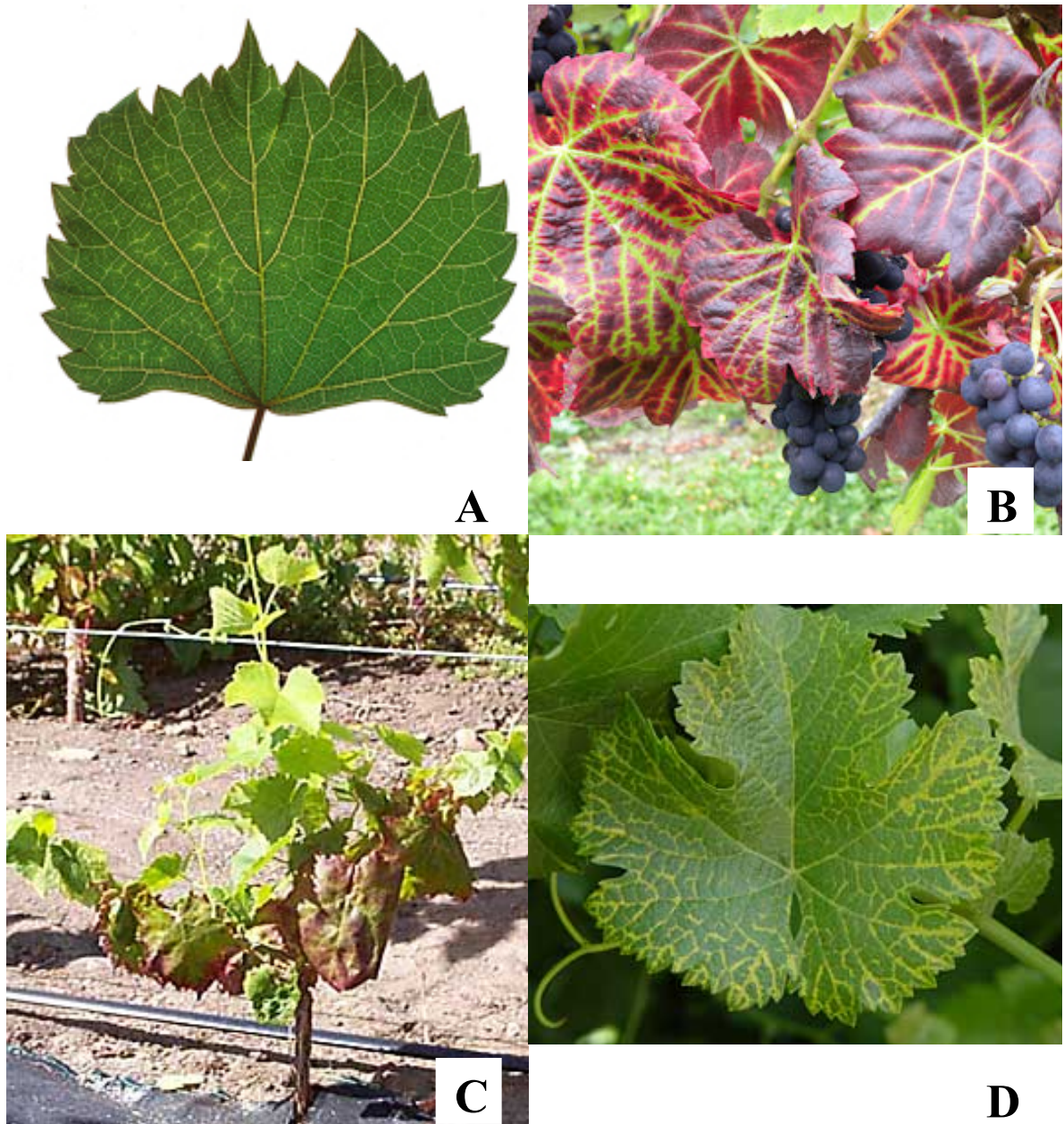


Figure 2. Characteristic symptoms of the RNA viruses of interest. A) Localized clearings, or “flecks”, in the veinlets of a young leaf as a result of *Grapevine fleck virus* (GFkV) infection. B) Slight downward rolling and discoloration of the leaves of a vine infected with *Grapevine leafroll-associated virus 3* (GLRaV-3). C) Rolling and reddening of the leaves of a vine infected with *Grapevine virus A* (GVA). The vine has also become severely stunted due to poor vigor. D) A leaf in which the primary veins have abnormally gathered due to *Grapevine fanleaf virus* (GFLV) infection, producing an open fan appearance of the leaf. GFLV is also responsible for the yellow vein-banding seen.

(Garau et al., 1994). It is a non-enveloped virus comprised of linear, positive-sense ssRNA. The genome is 7,349 nt long and encodes 5 ORFs (Galiakparov et al., 2003; Wang et al., 2012). Viral titer has been shown to also accumulate the most in the petioles of leaves, without regard to the leaf's age during Fall/Winter (Misbeh et al., 2007). Transmission of the virus can be done through propagation of infected plant material, but some mealybug species act as vectors as well. Rolling and reddening of the leaves of infected vines occurs similarly to that with GLRaV-3 except it is more severe and the discoloration affects the petioles also (Figure 2). Vines become severely stunted due to poor vigor, stems encounter cracking, and the cork under the bark develops pits and grooves. When infected vines are grafted, the graft union can swell and become necrotic (Pearson and Gohen, 1998).

GFLV, in the genus *Nepovirus* subgroup A, subfamily *Comovirinae*, family *Secoviridae* (2011), is considered the most serious viral disease on grapevines. It is a non-enveloped virus comprised of a segmented, bipartite genome comprised of two linear, positive-sense ssRNAs (Andret-Link et al., 2004). The genome consists of, RNA-1 of 7,342 nt and RNA-2 of 3,774-3,817 nt, and encodes two ORFs, one on each strand of RNA (Lamprecht et al., 2012; Tefera A. Mekuria, 2009). Like GFkV, viral titer accumulates mostly in the young leaves (Rowhani et al., 1993), except during Spring and Summer. GFLV is vectored by dagger nematodes (*Xiphinema index* and *X. italiae*) and transmitted by the propagation of infected plant material. The nominal symptom of GFLV is the abnormal gathering of primary veins, giving the leaves an open fan appearance (Figure 2). Leaves can also produce various chlorotic patterns (i.e. yellow

mosaic, yellow vein-banding). Other symptoms include a decreased winter hardiness, a reduction in fruit quality, and a drop in yield by up to 80% (Andret-Link et al., 2004).

In this study, three different grapevines are examined. Norton (*Vitis aestivalis*), also known as Cynthiana, is the oldest American grape variety commercially grown today and is the state grape of Missouri. It was first cultivated by Dr. Daniel Norton in Virginia in the 1830's (Ambers and Ambers, 2004). Norton is considered to be a “super” grape due to its resistance to a wide array of diseases, most notably powdery mildew (Fung et al., 2008). It has also shown little to no susceptibility to black rot, botrytis bunch rot, crown gall, and anthracnose (Domoto and Extension Fruit Specialist, 2008).

Chambourcin (*V. vinifera* hybrid) is a French hybrid cultivar of unknown parentage originally developed by Joannes Seyve along the Rhone (Galet, 1979). Despite having higher disease and winter resistance than *V. vinifera* cultivars (Dami), it is still susceptible to common, injurious pathogens. This includes black rot, botrytis bunch rot, and crown gall. It has shown susceptibility to downy mildew, as well (Domoto and Extension Fruit Specialist, 2008).

St. George (*V. rupestris*), or Rupestris du lot, is a shrub, as opposed to a vine, and is found in rocky to gravelly creek beds. Grazed almost to extinction, it is now a relatively rare species confined to streams in Northern Arkansas, Southern Missouri, and Tennessee. St. George is a stand-out variety because it roots and grafts easily and is able to tolerate drought due to a deep, extensive root system. However, it is most famous as one of the wild grapevines that saved Europe, especially France, from a plague of phylloxera in the 19th century thanks to its high resistance to the pest. It has also proven

to be successfully resistant to powdery mildew, downy mildew, root nematodes, Pierce's disease, black rot, and more (Gu, 2003).

Resistance to the aforementioned viral pathogens has not yet been examined in these cultivars until now. This study focuses on the four RNA viruses just mentioned, as well as the DNA virus, GVCV. Norton and Chambourcin were examined for potential resistance to GFkV, GLRaV-3, GVA, and GFLV. Norton and St. George were examined for potential resistance to GVCV. If any cultivar proves resistant to one or more viruses, it could be used to integrate its resistance into more susceptible varieties by either conventional breeding techniques or biotechnology.

Further work was done with GVCV upon observation of vein clearing and vine decline symptoms synonymous with GVCV on wild *V. rupestris* vines. Samples of the infected vine were collected, tested, and found to be infected with GVCV. Until this discovery GVCV had only been found in cultivated grapevines. Therefore, the genome of GVCV was sequenced to determine whether any genetic differences exist between the two viruses infecting wild and cultivated vines.

CHAPTER 1: ASSESSMENT OF THREE GRAPE VARIETIES' RESISTANCE TO FIVE VIRUSES

Introduction

Grapevine fleck virus (GFkV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine virus A* (GVA), and *Grapevine fanleaf virus* (GFLV) are the most widespread grapevine viruses that are major viral pathogens to grape and wine industries worldwide. GLRaV-3, GVA, and GFLV, in particular, can become greatly debilitating to vineyards (Gambino and Gribaudo, 2006). The recently characterized *Grapevine vein clearing virus* (GVCV) has also proven to be a serious concern, specifically to Midwestern vineyards (Guo et al., 2013; Zhang et al., 2011). It is vital to identify cultivars resistant to these viral diseases in order to attempt to prevent infection by these viruses and protect commercial vineyards from yield and economic loss. A cultivar that proves resistant to one or more virus can potentially be utilized as a parent in breeding in an attempt to confer this resistance to susceptible vines. Conventional breeding techniques and biotechnology can be used to integrate the resistant gene into the genome of susceptible varieties. If successful, this would result in less damage to and loss of vines, producing higher crop yield and greater quality, which, in turn, would lead to more economic return and ecological and environmental benefits.

Norton (*Vitis aestivalis*) and Chambourcin (*Vitis vinifera* hybrid) were examined for potential resistance to GFkV, GLRaV-3, GVA, and GFLV. Norton and St. George (*Vitis rupestris*) were examined for potential resistance to GVCV. St. George was not used in testing of GFkV, GLRaV-3, GVA, or GFLV because it is known to be susceptible

to these viruses and used as indicator plant for bio-indexing of grapevine virus infection. However, because symptoms synonymous with GVCV infection had been observed on wild *V. rupestris* bushes, St. George was used in testing of GVCV. Chambourcin was not used in testing of GVCV because it has already proven to be resistant to GVCV in the previous study of the virus in our laboratory (Guo et al., 2013).

Materials and Methods

Virus-infected and Virus-free Vines. Green softwood cuttings were taken from Norton, Chambourcin, St. George, Kishmish vatkana (*Vitis vinifera*) (KV), and Chardonel (*Vitis vinifera* hybrid) vines grown under greenhouse conditions at the Missouri State Fruit Experiment Station, Mountain Grove, Missouri, USA. The Norton, Chambourcin, and St. George vines were previously tested as virus-free. The KV and Chardonel vines were previously infected with their respectable viruses and tested positive for these viruses, and thus were used as sources of viruses. The KV vine was infected with a mixed population of the four RNA viruses of interest. The Chardonel LBC0903 was infected with GVCV.

Inoculation of Grapevines with Viruses by Graft-transmission. Cuttings of one node (scion) were taken from the virus-infected vines and grafted onto cuttings of two nodes (rootstock) from the virus-free vines. Three replicates were performed for each rootstock/scion combination. All grafts were done by the wedge-grafting technique.

Virus-free Norton and Chambourcin rootstocks were grafted with virus-infected KV scions. For GVCV testing, virus-free Norton and St. George rootstocks were grafted

with GVCV-infected Chardonnay scions. A single reciprocal graft was performed for Norton (i.e. virus-free Norton scion grafted onto GVCV-infected Chardonnay rootstock).

The fresh-cut ends of the rootstocks were dipped in Rhizopon AA #1 rooting powder (The Hortus USA Corp, Earth City, MO, USA) and placed in sponge plugs in a Styrofoam tray. The grafted vines were kept under mist in a greenhouse until rooted. After being transferred to potted soil, they were kept under greenhouse conditions for several months to allow the graft unions to be completely healed and for the viral infections to spread into the rootstocks.

Initially, results were inconclusive for GFLV. The virus-free Norton mother vines were too woody at the time to repeat the experiment for this cultivar, but new grafts were able to be produced for Chambourcin. The virus-infected cuttings were taken from a St. George vine (sGFLV) which had previously tested positive for GFLV infection. Reciprocal grafts were made for Chambourcin as well (i.e. virus-free Chambourcin scion grafted onto virus-infected sGFLV rootstock). A reciprocal graft using virus-free Chardonnay (*Vitis vinifera*) was also done to act as an isolated control. Chardonnay was chosen because it is already known to be susceptible to GFLV (Tefera A. Mekuria, 2009). Testing was performed as described below.

Tissue Sampling and Extraction of RNA and DNA. RNA was extracted from leaf samples of both the rootstocks and the scions of the grafted vines using the Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Ontario, Canada). The type of leaf tissue used and the timing of extraction was determined in a manner to obtain the highest titer of each individual virus, as described in Chapter 1. DNA was extracted from young leaf tissue of both the rootstocks and scions of the

grafted vines using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA). For each samples, 100 mg of fresh tissue was ground into a fine powder in liquid nitrogen by using a mortar and pestle.

Analysis of RNA and DNA Quality and Quantity. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to analyze the quality and concentration of the extracted RNA. A NanoDrop 2000 Spectrophotometer (NanoDrop Products, Wilmington, DE, USA) was used to measure the concentration of the extracted DNA. All DNA samples were diluted to 10 ng/ μ L.

Reverse Transcription (RT)-PCR Assay of RNA Viruses. After analysis with the Agilent 2100 Bioanalyzer, 3 μ g of RNA from each sample was added in a 20 μ L solution to make cDNA following the Superscript III Reverse Transcriptase protocol as provided by Invitrogen™ (Life Technologies, Carlsbad, CA, USA). The cDNA quality was analyzed via polymerase chain reaction (PCR) using 10 μ M 18S internal control primers (Table 1) developed by Gambino and Gribaudo (2006), 10 mM dNTP mix, and 5X GoTaq® reaction buffer and DNA polymerase (Promega Corp., Madison, WI, USA). 1 μ L of cDNA solution was used for each reaction. Primer sets specific to each virus were list in Table 1. For the KV grafts, samples were tested using primers for GFkV, GLRaV-3, GVA, and GFLV, individually. Results were analyzed by running 10 μ L PCR product on a 1% agarose gel stained with GelRed™ nucleic acid gel stain (Biotium Inc., Hayward, CA, USA) and were visualized with a GelDoc-It Imaging System (UVP LLC., Upland, CA, USA).

Table 1. A list of primers utilized in the PCR testing of virus infection. Primer sequences for 18S rRNA, *Grapevine fleck virus* (GFkV), and *Grapevine leafroll-associated virus 3* (GLRaV-3) were acquired from the study by Gambino and Gribaudo (2006). Primer sequences for *Grapevine virus A* (GVA) were designed by Goszczynski and Jooste (2003). Primer sequences for *Grapevine fanleaf virus* (GFLV) and *Grapevine vein clearing virus* (GVCV) were designed in the laboratory.

Primer	Detection	Sequence (5'-3')	T _a (°C)	Size (bp)
18S F 18S R	18S rRNA	CGCATCATTCAAATTTCTGC TTCAGCCTTGCGACCATACT	52.0	844
GFkV F GFkV R	GFkV	TGACCAGCCTGCTGTCTCTA TGGACAGGGAGGTGTAGGAG	57.0	179
GLRaV-3 F GLRaV-3 R	GLRaV-3	TACGTTAAGGACGGGACACAGG TGCGGCATTAATCTTCATTG	53.0	336
GVA F GVA R	GVA	GAGGTAGATATAGTAGGACCTA TCGAACATAACCTGTGGCTC	54.0	272
GFLV5 F GFLV-VC1 R	GFLV	GCTGCAATGATTGCYTGycATGG GACGGGAARCTGGTTCTWCC	59.0	354
GVCV2460 F GVCV3122 R	GVCV	AGACACAGGAGAAAGGGTAACT GCTAAAACCTTTCGAGCTAAC	52.3	663
GVCV1101 F GVCV1935 R	GVCV	CTGAAAGGTAGATGTCCACG TCGGTGTAGCACTTGTATTCT	54.7	835

PCR Assay for GVCV Infection. The presence of GVCV-specific DNA within the samples was determined via PCR, which was performed in the same procedures as previously discussed. In testing for GVCV, 1 μ L of DNA solution (10 ng) was used as template. For the Chardonel grafts, samples were tested using primers for GVCV. As before, 10 μ L of PCR solution was run on a 1% agarose gel, stained with GelRed, and the results were visualized under ultraviolet light.

Results

Cuttings from the KV vine, a grapevine infected with GFkV, GLRaV-3, GVA, and GFLV, were grafted onto virus-free Norton and Chambourcin rootstocks and the graft unions were allowed to heal. About 6 months later, RNA was extracted from leaf tissue of both the rootstocks and scions. The RNA was then synthesized into cDNA and proven to be of acceptable quality for testing via RT-PCR assay of grapevine 18S rRNA. Samples were then examined for viral infection through multiple PCR assays using one pair of specific primers to each virus.

In the KV/Norton grafts, GFkV was detected in both the scion and rootstock samples (Figure 3). A band was expected to be present in the scion, as it had already been shown to be infected, but the presence of GFkV in the rootstock demonstrated that this virus is able to transfer from the KV scion to the Norton rootstock. Therefore, Norton is susceptible to GFkV infection. In the KV/Chambourcin grafts, GFkV was once again detected in both the scion, as expected, and the rootstock samples (Figure 3), demonstrating the ability of the virus to transfer from the KV scion to the Chambourcin rootstock. Therefore, Chambourcin is also susceptible to GFkV infection.

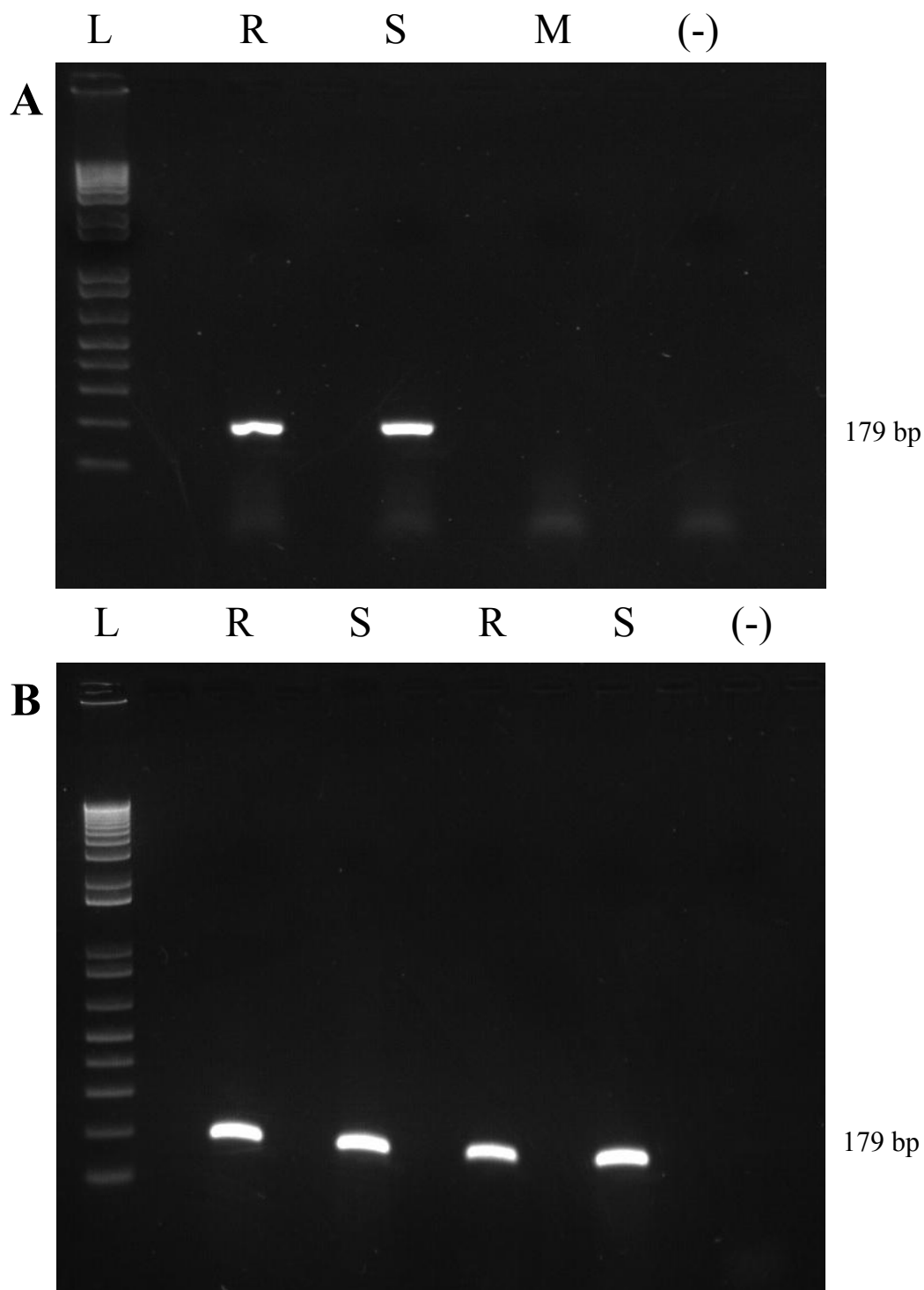


Figure 3. Testing for infection of *Grapevine fleck virus* (GFkV) in A) Norton (*Vitis aestivalis*) and B) Chambourcin (*Vitis vinifera* hybrid). A) Lane L = 1 Kb ladder; Lane R = Norton Rootstock; Lane S = KV Scion; Lane M = Virus-Free Norton Mother Vine; Lane (-) = Negative Control. B) Lane L = 1 Kb Ladder; Lanes R = Chambourcin Rootstocks; Lanes S = KV Scions; Lane (-) = Negative Control. The bands present at about 179 bp are GFkV-specific.

Testing for GLRaV-3 and GVA produced similar results for both Norton and Chambourcin. In the KV/Norton grafts, both GLRaV-3 (Figure 4) and GVA (Figure 5) were detected in the scion and the rootstock samples. This was true for the KV/Chambourcin grafts, also. Both viruses, then are able to transfer from the KV scion to the Norton and Chambourcin rootstocks, indicating that the two varieties are susceptible to GLRaV-3 and GVA.

Due to issues amplifying GFLV fragments, new grafts were developed for Chambourcin using a GFLV-infected St. George vine as the scion. A reciprocal graft was also developed. RNA was extracted and cDNA synthesized and tested for infection following the same protocols. In the sGFLV/Chambourcin grafting combinations, GFLV was detected in the scion and the rootstock samples from both the standard grafts as well as the reciprocal graft (Figure 6). These results demonstrate that GFLV, like the three other RNA viruses, is able to transfer from the sGFLV scion to the Chambourcin rootstock. These results also show the ability of the virus to transfer from the sGFLV rootstock up to the Chambourcin scion. The band intensity indicative of GFLV infection in the Chambourcin scion, though, appears much lighter than those seen in the Chambourcin rootstock and both sGFLV samples. This could potentially be due to the viral titer in the Chambourcin scion being much lower than that of the other samples, but more work is necessary to confirm this. Nonetheless, Chambourcin is still susceptible as both a rootstock and a scion.

Cuttings from a Chardonnay grapevine 'LBC0903' infected with GVCV were grafted onto virus-free Norton and St. George rootstocks and allowed to heal. DNA was

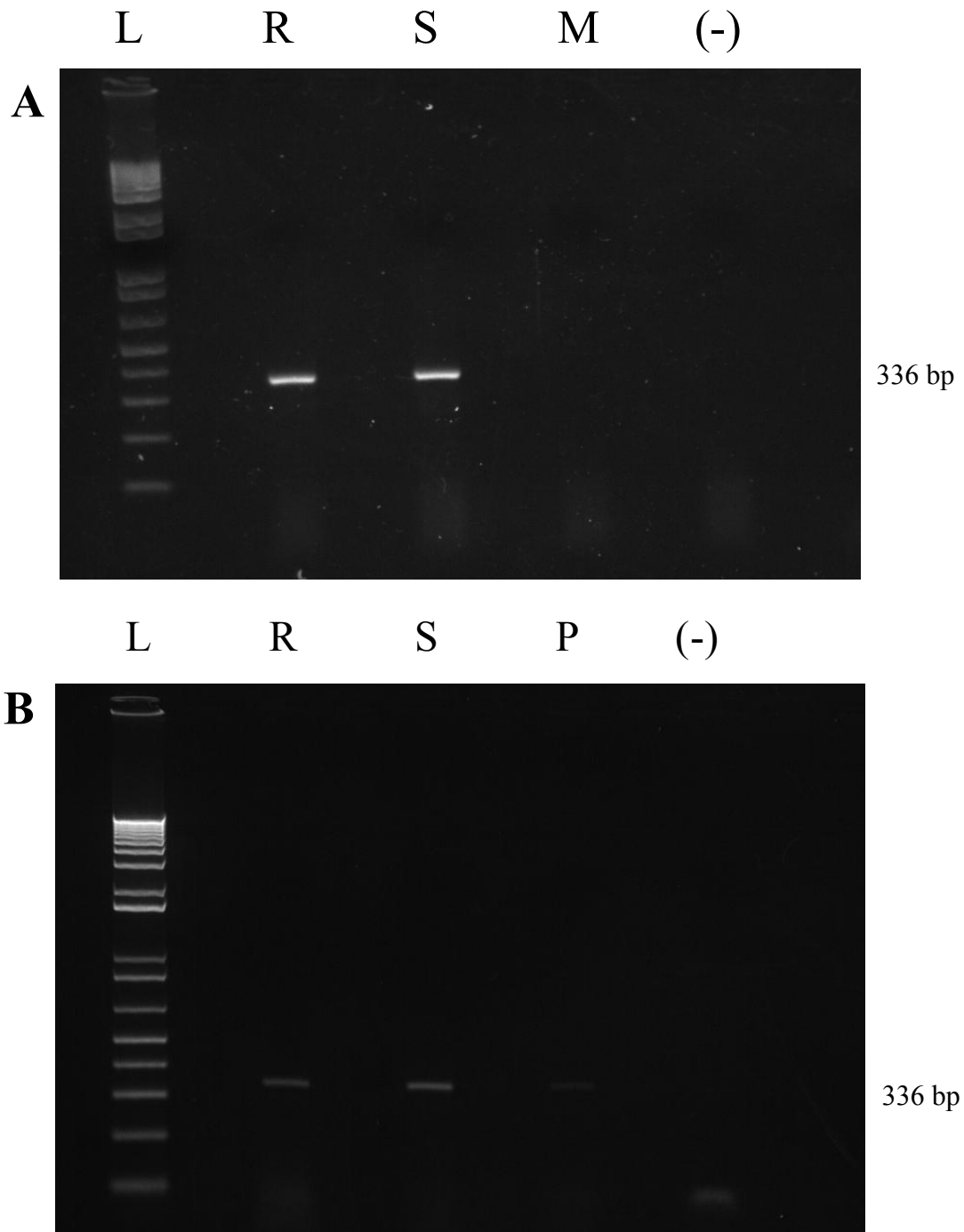


Figure 4. Testing for infection of *Grapevine leafroll-associated virus 3* (GLRaV-3) in A) Norton (*Vitis aestivalis*) and B) Chambourcin (*Vitis vinifera* hybrid). A) Lane L = 1 Kb ladder; Lane R = Norton Rootstock; Lane S = KV Scion; Lane M = Virus-Free Norton Mother Vine; Lane (-) = Negative Control. B) Lane L = 1 Kb ladder; Lane R = Chambourcin Rootstock; Lane S = KV Scion; Lane P = Virus-Infected KV vine; Lane (-) = Negative Control. The 336 bp DNA fragment is amplified by GLRaV-3 specific primers.

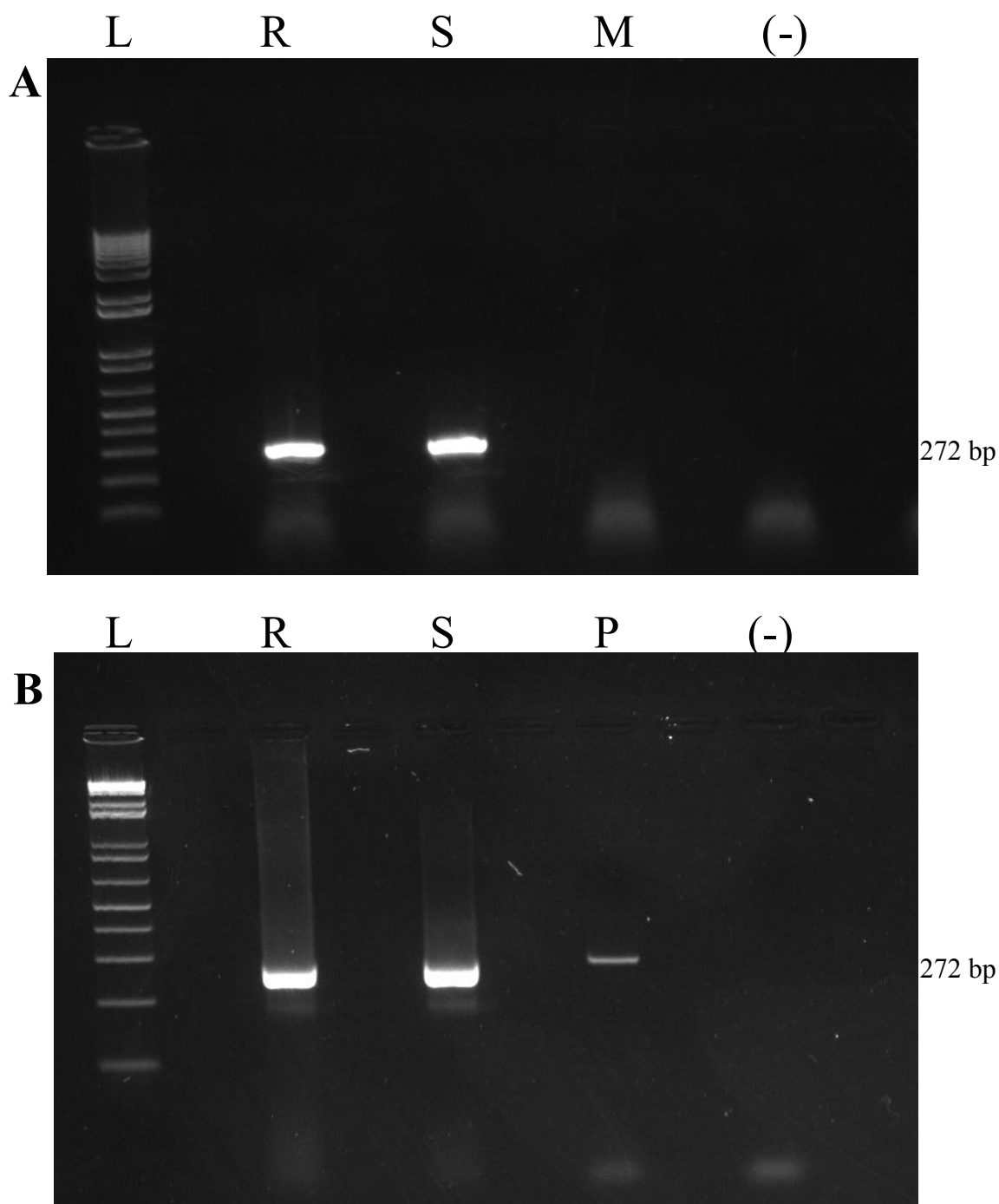


Figure 5. Testing for infection of *Grapevine virus A* (GVA) in A) Norton (*Vitis aestivalis*) and B) Chambourcin (*Vitis vinifera* hybrid). A) Lane L = 1 Kb ladder; Lane R = Norton Rootstock; Lane S = KV Scion; Lane M = Virus-Free Norton Mother Vine; Lane (-) = Negative Control. B) Lane L = 1 Kb ladder; Lane R = Chambourcin Rootstock; Lane S = KV Scion; Lane P = Virus-Infected KV Mother Vine; Lane (-) = Negative Control. The 272 bp DNA band is GVA-specific.

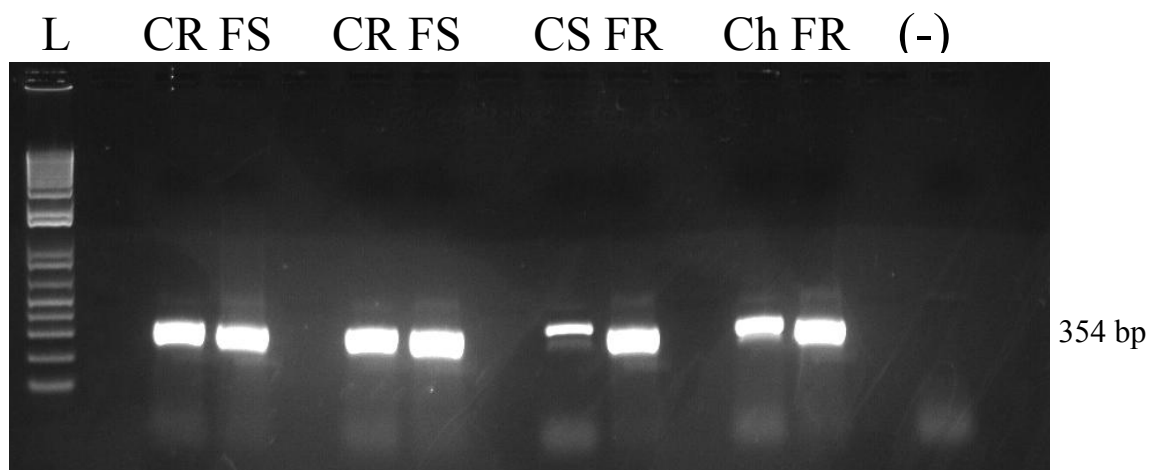


Figure 6. Testing for infection of *Grapevine fanleaf virus* (GFLV) in Chambourcin (*Vitis vinifera* hybrid). Lane L = 1 Kb Ladder; Lanes CR = Chambourcin Rootstock; Lanes FS = sGFLV Scion; Lane CS = Chambourcin Scion; Lanes FR = sGFLV Rootstock; Lane Ch = Chardonnay Control Scion; Lane (-) = Negative Control. The 354 bp band is amplified by GFLV-specific primers.

extracted from leaf tissue of both the rootstocks and scions. Samples were then examined for viral infection through PCR assays by using GVCV-specific primers.

In the Chardonnay LBC0903/Norton grafts, GVCV was detected in both the scion and rootstock samples (Figure 7). The presence of GVCV in the rootstock demonstrates that this virus is able to transfer from the Chardonnay scion to the Norton rootstock.

However, in the Chardonnay LBC0903/Norton reciprocal graft, GVCV was detected in the rootstock sample, but not in the scion sample (Figure 8). This result demonstrated that Norton is resistant to GVCV infection when grafted in the scion position, which will be an interesting phenomena for future investigation. Therefore, Norton is susceptible to GVCV infection as a rootstock, but potentially resistant as a scion. In the Chardonnay

LBC0903/St. George grafts, GVCV was detected in the scion and rootstock samples, demonstrating the capability of GVCV to transfer from the Chardonnay scion to the St. George rootstock. Therefore, St. George is susceptible to GVCV infection.

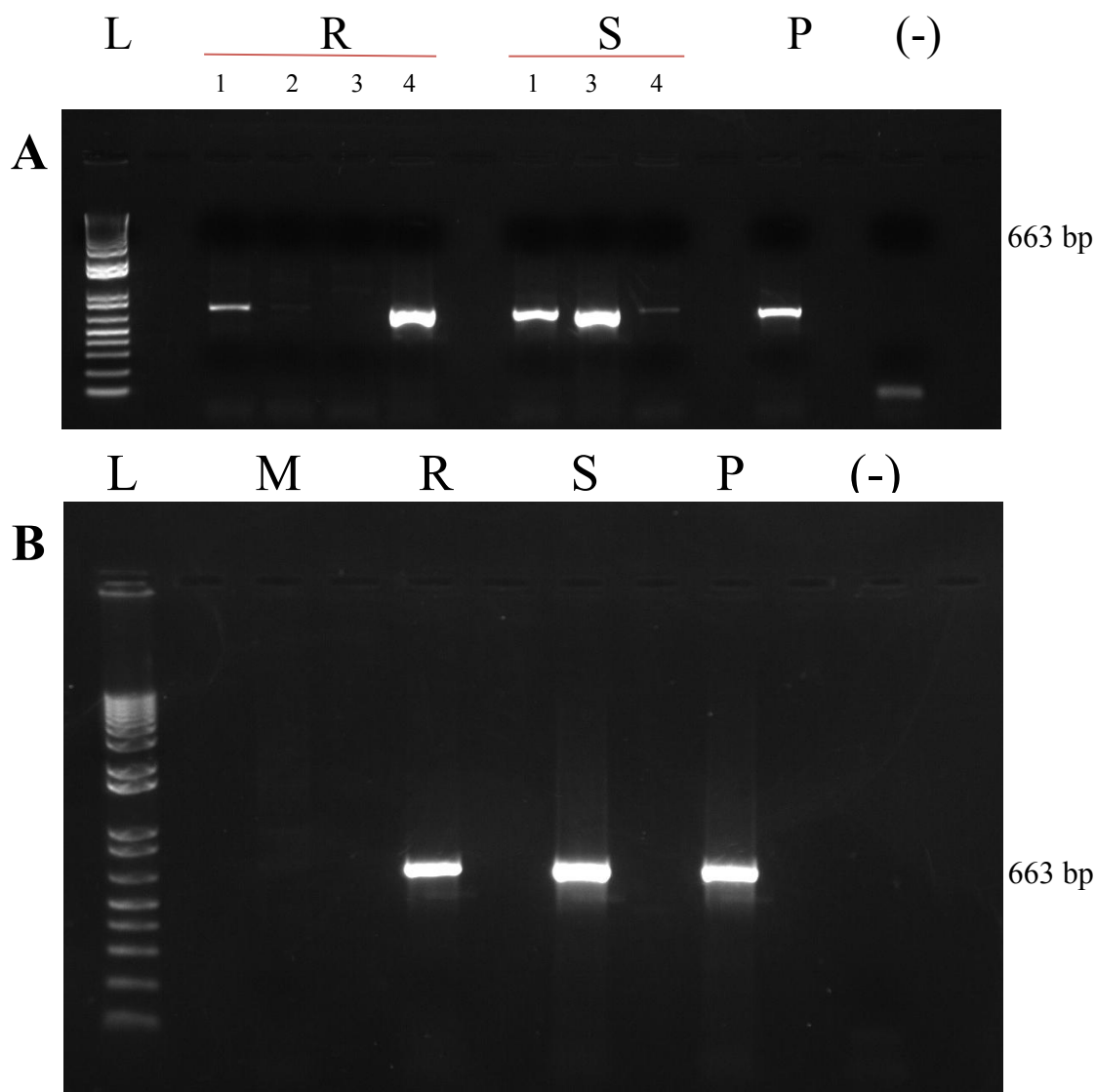


Figure 7. Testing for infection of *Grapevine vein clearing virus* (GVCV) in A) Norton (*Vitis aestivalis*) and B) St. George (*Vitis rupestris*). A) Lane L = 1 Kb ladder; Lanes R = Norton Rootstocks; Lanes S = Chardonel Scions; Lane P = Virus-Infected Chardonel Mother Vine; Lane (-) = Negative Control. There is no S2 due to the scion dying before samples could be taken. The faint band in R2, though, shows that the virus was able to be transmitted to the Norton rootstock within the short period they were grafted before the Chardonel scion died. B) Lane L = 1 Kb ladder; Lane M = Virus-Free St. George Mother Vine; Lane R = St. George Rootstock; Lane S = Chardonel Scion; Lane P = Virus-Infected Chardonel Mother Vine; Lane (-) = Negative Control. The 663 bp band is GVCV-specific.

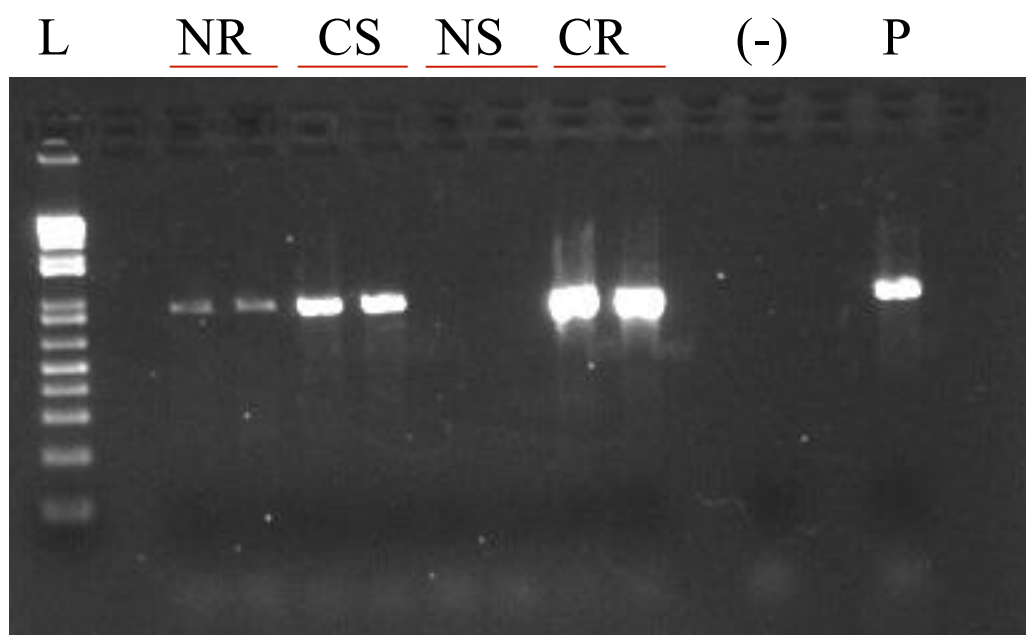


Figure 8. Testing for infection of *Grapevine vein clearing virus* (GVCV) in the Norton reciprocal grafting combinations. Lane L = 1 Kb ladder; Lanes NR = Norton Rootstock; Lanes CS = Chardonnay LBC0903 Scion; Lanes NS = Norton Scion; Lanes CR = Chardonnay LBC0903 Rootstock; Lane (-) = Negative Control Lane P = Virus-Infected Chardonnay Mother Vine. The bands present at about 835 bp in NR supports the results in Figure 7 indicating Norton to be susceptible to GVCV infection when grafted as a rootstock. The absence of bands in NS demonstrate possible resistance to GVCV infection in Norton when it is grafted as a scion.

Discussion

Since Chambourcin showed susceptibility to all four viruses tested, it should not be used in vineyards in attempt to prevent any of these diseases. When infected with GFLV, Chambourcin showed susceptibility not only as a rootstock, but as a scion as well. Under visual observation, the virus-specific DNA band in the Chambourcin scion appeared less concentrated than that produced from the Chambourcin rootstock, indicating the GFLV titer is lower in the Chambourcin scion than in the Chambourcin rootstock. Quantitative PCR (qPCR) would need to be performed to confirm this. As only one graft was examined in which Chambourcin was the scion, its tolerance to GFLV as a scion cannot be firmed until more replicates are examined. If the results are verified

after further testing, it would be interesting to investigate the mechanism on how Chambourcin is able to reduce viral titer when grafted as a scion. Furthermore, is there any way to take advantage of this special trait to reduce the rate of infection in Chambourcin scions, such as the addition of an interstock to the graft?

Despite its resistance to a multitude of fungal and bacterial pathogens, Norton was found to be susceptible to all viruses examined, with the exception of GFLV, for which no conclusive results were obtained. Norton, therefore, should not be used in the breeding program in attempt to acquire virus-resistant new grape cultivars. However, Norton showed resistance to GVCV when grafted as a scion. Only one graft was examined in which Norton was the scion, though, so more replicates are required before it is concluded that Norton is resistant as a scion. If these results are verified after further testing, it would be interesting to explore how Norton is able to block GVCV infection when grafted as a scion and why it is unable to do this when grafted as a rootstock.

St. George also proved susceptible to GVCV and should not be used in vineyards to attempt to control this virus. Just like with Norton, these results are surprising due to St. George's history of high resistance. It is interesting to see how two grape varieties that are commonly utilized for their ability to resist disease by some of the most common and debilitating pathogens are still susceptible to viral infection. Yet Chambourcin, the cultivar considered the most disease susceptible of the three, shows resistance to GVCV (Guo et al., 2013).

In conclusion, it is apparently more difficult for grapevines to develop resistance to viruses than to other pathogens. Chambourcin proved susceptible to infection by all four RNA viruses, GFkV, GLRaV-3, GVA, and GFLV. This is not too surprising,

though, as Chambourcin has shown to be susceptible to a multitude of infections (Domoto and Extension Fruit Specialist, 2008). Norton, however, also proved susceptible to all four RNA viruses, as well as the DNA virus, GVCV. This is surprising since Norton has shown resistance to so many other pathogens in the past, although the diseases Norton is resistant to are mainly fungi and bacteria, not viruses (Domoto and Extension Fruit Specialist, 2008). The results did indicate a potential resistance to GVCV infection when Norton is grafted in the scion position, but further research will be needed to confirm this observation. St. George is a variety of grapevine commonly used as a rootstock in vineyards because of its disease resistance, particularly to phylloxera (Gu, 2003), yet it also was determined to be susceptible to GVCV infection. If a grape grower is looking to protect their vineyard from either of these four RNA viruses studied, then they should not rely on Chambourcin or Norton to protect their crops. Their best option would probably be to defend against vectors and to periodically test their vines, removing any that test positive for infection. Chambourcin is resistant to GVCV (Guo et al., 2013), though, and can be utilized to instill resistance in more susceptible varieties of a vineyard. However, Norton and St. George cannot. So if a grape grower is looking to protect their vineyard from GVCV, their best option would be to either use Chambourcin or one of the other methods previously mentioned.

CHAPTER 2: ASSEMBLY OF A NOVEL GRAPEVINE VEIN CLEARING VIRUS GENOME

Introduction

Grapevine vein clearing virus (GVCV) is a DNA virus associated with a severe vein clearing and vine decline disease in cultivated grapevines. GVCV-associated disease was first observed in 2004, but the virus was discovered as a novel virus in 2011 (Zhang et al., 2011). Notable symptoms include short, zig-zagged internodes, as well as translucent vein-clearing and backward rolling of the leaves with chlorotic mosaic patterns.

Wild *Vitis rupestris* ‘Scheele’ shrubs expressing GVCV-like symptoms were recently observed growing along the bank of Swan Creek in Taney County, MO. In addition, necrotic flecks were observed along the cleared veins. Cuttings from the diseased vine were taken and propagated in potted soil in the greenhouse at the Missouri State Fruit Experiment Station, Mountain Grove, MO, USA. They were tested and confirmed to be infected with GVCV, marking the first instance that this virus has been found in the wild grapevine. This is also the first time that necrotic flecks have been observed along the cleared veins of an infected vine. Therefore, the flecks were either caused by another pathogen present in the ‘Scheele’ or they were caused by the GVCV infection. Because these flecks had never been observed before, though, if they were caused by GVCV then it makes sense that the GVCV in the wild must somehow differ from the GVCV in the cultivated vines.

It is unknown if the GVCV found in the wild *V. rupestris*, GVCV-VRU, is the same as the first GVCV virus at the genome sequence level. The entire genome of GVCV-VRU was sequenced and compared with the reference genome of GVCV-CHA. The genome of GVCV-VRU was found to be 7,755 bp long with three ORFs. Sharing less than 92% nucleotide identity to GVCV-CHA, GVCV-VRU was defined as a new isolate of GVCV.

Materials and Methods

Collection of Wild Vines. Accessions of *V. rupestris* ‘Scheele’ exhibiting GVCV-like symptoms were collected from the bank of Swan creek in Taney County, MO, USA. Cuttings were placed into a plastic bag with a moist paper towel and put on ice for transport to the laboratory, where they were stored at 4°C. They were rooted, planted in potted soil, and grown under greenhouse conditions at the Missouri State Fruit Experiment Station, Mountain Grove, MO, USA.

Extraction of DNA. About 100 mg of leaf tissue was sampled from the propagated canes (sample name VRU89) and frozen in liquid nitrogen. The tissue was ground into a fine powder by mortar & pestle and total DNA extracted using the Qiagen DNeasy Mini Plant Kit (Qiagen Inc., Valencia, CA, USA). DNA was eluted in 50µL of dH₂O and the concentration was measured using a NanoDrop 2000 Spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

Polymerase Chain Reactions and Electrophoresis. Presence of GVCV was first confirmed in VRU89 by polymerase chain reaction (PCR) using the GVCV-specific primer sets in Table 1. The PCR assay was performed as instructed by the protocol

provided by Life Technologies TM (Grand Island, NY, USA). Master mix was composed of 1× High Fidelity Platinum *Taq* buffer, 2 mM MgCl₂, 0.2 mM dNTP mix, 0.2 μM primers, 25 ng template DNA, and 1 unit Platinum® High Fidelity *Taq* DNA Polymerase. Thermocycler conditions were as follows: initial denaturation for 1 minute at 94°C; 35 cycles of denaturation at 94°C for 30 seconds, annealing at a temperature specific for each set of primer for 40 s, and extension at 72°C with time determined by the nucleotide length of each fragment; final extension at 72°C for 10 min. 10 μL PCR products were run on a 1% agarose gel stained with GelRedTM nucleic acid gel stain (Biotium Inc., Hayward, CA, USA) in 1X Tris-Borate EDTA buffer and were visualized with a GelDoc-It Imaging System (UVP LLC., Upland, CA, USA).

PCR was repeated on VRU89 to amplify three overlapping fragments, covering the entire viral genome, using the primers listed in Table 2. The primer sets were designed from the genomic sequence of the original GVCV genome previously observed in cultivated vines and presented in Figure 9. The assay was performed and analyzed in the same manner as described in the previous section.

DNA Purification and Cloning. Using the MinElute Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA), the three PCR-amplified DNA fragments were purified from the agarose gel. They were then inserted into the pCRTM8/GW/TOPO[®] vector plasmid using the Gateway TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Ligation was performed in 6 μl reaction composed of 0.5-4 μl of purified DNA fragment, 1 μl of salt solution, 1 μl of TOPO[®] vector plasmid DNA and sterile dH₂O following the guidelines of TOPO TA Cloning Kit. Recombinant pCRTM8/GW/TOPO[®] constructs were transformed into chemically competent One Shot[®] TOP10 *E. coli* cells by heat-shock.

Table 2. Primers utilized in the cloning and sequencing the genome of GVCV-VRU isolate. Primer sequences were developed by Zhang et al. (2011).

Primer	Sequence (5'-3')	T_a (°C)	Size (bp)
GVCV1915	AGAATACAAGTGCTACACCGA	54.7	2248
GVCV4162	CATGAGAGTCATGAGGTTTAC		
GVCV4142	GTAAACCTCATGACTCTCATG	54.7	2654
GVCV6795	GCTGGCGTAAGCACAGATTC		
GVCV6666	ACTTCCTCCACCCCACGCAGTTATC	54.7	3023
GVCV1935	TCGGTGTAGCACTTGTATTCT		

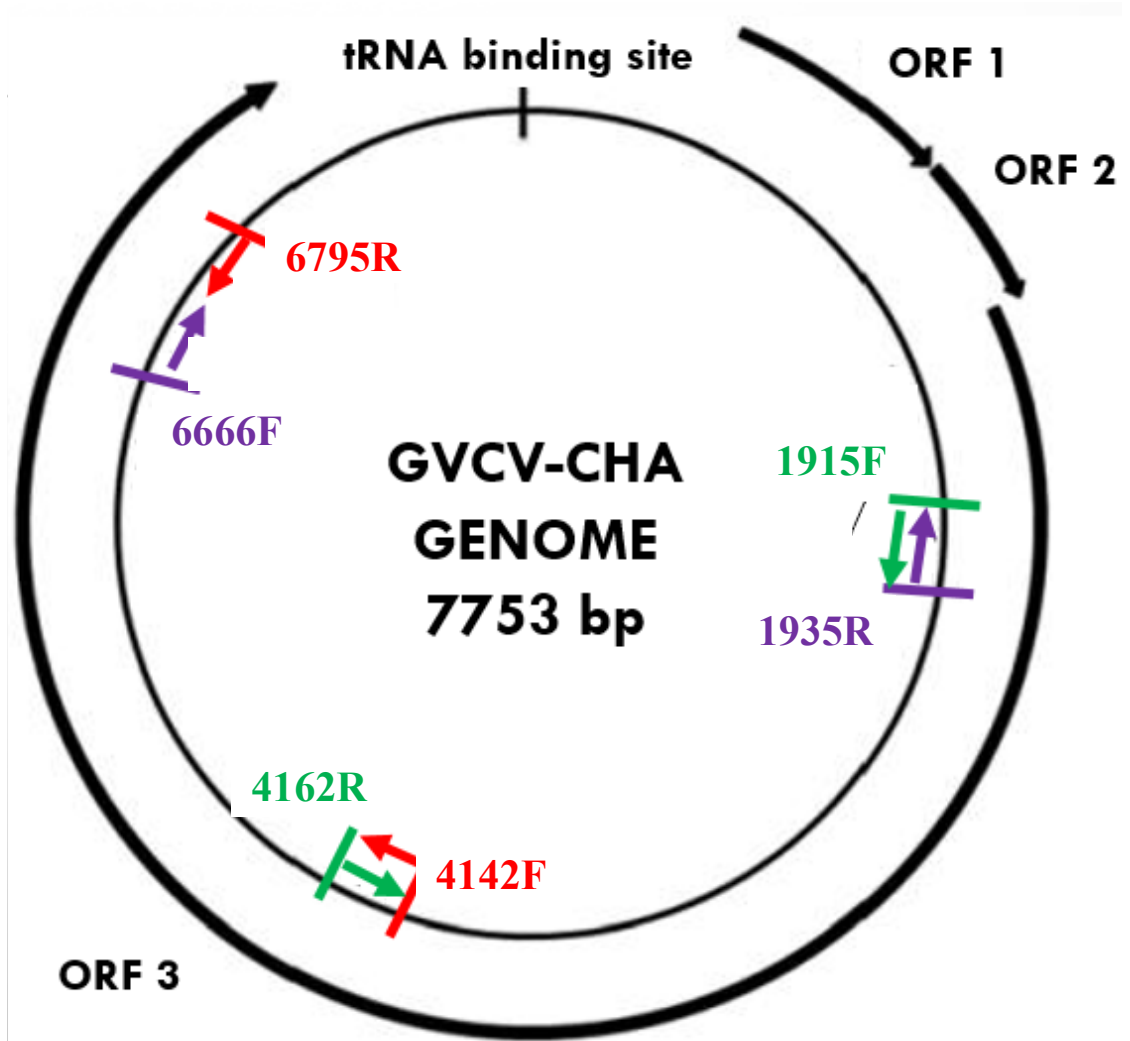


Figure 9. The three overlapping fragments amplified by PCR for sequencing the genome of the GVCV-VRU isolate infecting wild *Vitis rupestris* 'Scheele'. The three fragments and their corresponding primer sets for amplification were designed from the original GVCV-CHA genome. The first fragment extends from 1915 to 4162 bp. The second fragment extends from 4142 to 6795 bp. The third and final fragment extends from 6666 to 1935 bp.

The bacteria transformed with recombinant plasmids were cultured on sterilized Luria-Bertani (LB) agar medium plates with spectinomycin at a concentration of 100 µg/ml in a 37°C incubator. Two individual colonies were selected and cultured in liquid LB medium with spectinomycin in a 37°C shaker overnight at 220rpm. Recombinant plasmid DNA was isolated using QIAprep® Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA) and confirmed to contain the GVCV fragment by PCR with GVCV-specific primers as well as universal GW1 primer (5'-GTTGCAACAAATTGATGAGCAATGC-3') and GW2 primer (5'-GTTGCAACAAATTGATGAGCAATTA-3'). chemically competent One Shot® TOP10 *E. coli* cells by heat-shock. The bacteria transformed with recombinant plasmids were cultured on sterilized Luria-Bertani (LB) agar medium plates with spectinomycin at a concentration of 100 µg/ml in a 37°C incubator. Two individual colonies were selected and cultured in liquid LB medium with spectinomycin in a 37°C shaker overnight at 220rpm. Recombinant plasmid DNA was isolated using QIAprep® Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA) and confirmed to contain the GVCV fragment by PCR with GVCV-specific primers as well as universal GW1 primer (5'-GTTGCAACAAATTGATGAGCAATGC-3') and GW2 primer (5'-GTTGCAACAAATTGATGAGCAATTA-3').

Sequence Assembly and Analysis. The recombinant plasmid constructs were sent to Eurofins MWG Operon DNA Sequencing Facility (Huntsville, AL, USA) where they were sequenced in both direction using GW1 and GW2 primers. The primer-walking method was utilized to acquire the nucleotide sequence of the entire insert. The Vector NTI program (Invitrogen™) was used to assemble and analyze the sequence results.

Results

Symptoms of GVCV were observed in the wild for the first time on *V. rupestris* ‘Scheele’. PCR testing confirmed GVCV infection in the wild grapevine ‘VRU89’. The reference genome sequence of GVCV previously observed in cultivated vines was used for designing primers to amplify DNA fragments from the VRU89 sample by PCR. Three fragments were amplified that covered the entire GVCV genome with overlapping regions.

The fragments were sequenced and assembled into a complete genome of what was found to be a novel GVCV isolate. This new isolate was referred to as GVCV-VRU, since it was found in *V. rupestris*, and the reference isolate has been distinguished as GVCV-CHA, since it was first identified in a Chardonnay vine. GVCV-VRU genome is 7,755 bp in length, 2 bp longer than that of GVCV-CHA, and the two isolates share 91.5% identity at the nucleotide level. Difference between the two sequences is attributed to single nucleotide polymorphisms (SNPs) and 38 indels.

Three ORFs were predicted on the plus-strand of GVCV-VRU, indicating that it had the same coding regions as GVCV-CHA. Identity of ORF I-encoded proteins between the two isolates was 97.6% with a three-amino acid difference. The ORF II-encoded proteins differed in eighteen amino acids between the two isolates, and the overall amino acid sequence identity was 86.2%. A total of 70 amino acids differed in the ORF III-encoded proteins of the genomes of the two isolates with 96.2% overall identity. Within the ORF III protein, the amino acid sequence identity of the RT domain was 99.4% while that of the RNase H domain was 98.5% between the two isolates (Table 3).

The ORF II region was the most divergent between GVCV-CHA and GVCV-VRU. The ORF II of GVCV-CHA was 381 nt in length while the ORF II of GVCV-VRU had 390 nt with overall identity of 83.3% at the nucleotide sequence level. The difference in length was due to a 9 nt indel starting at nucleotide 1,373. Sequencing of the 835 bp fragment covering the ORF II in additional recombinant plasmids confirmed the nucleotide difference between the two isolates. At the amino acid level, there is 86.2% identity with the extra nucleotides encoding for a SKA amino acid sequence in GVCV-VRU that is absent in GVCV-CHA.

Table 3. Comparative size and sequence identity of the whole genome and three ORFs of the two *Grapevine vein clearing virus* (GVCV) isolates.

Genome Segment		GVCV-CHA	GVCV-VRU	Identity
Total nt		7,753	7,755	91.5%
ORF I	Start-Stop Length	485-1,111 (627 nt)	484-1,110 (627 nt)	94%
	Amino Acids	209	209	97.6%
ORF II	Start-Stop Length	1,112-1,495 (384 nt)	1,111-1,503 (393 nt)	83.3%
	Amino Acids	128	131	86.2%
ORF III	Start-Stop Length	1,495-7,320 (5,826 nt)	1,503-7,328 (5,826 nt)	92%
	Amino Acids	1,942	1,942	96.2%

The first 12 nucleotides of both the GVCV-CHA genome (5'-TGGTATCAGAGCtccag) and the GVCV-VRU genome (5'-TGGTATCAGAGCcag) were complementary to the 3'-end 12 nucleotides of the plant tRNA^{MET} consensus sequences (3'-ACCAUAGUCUCGguccaa-5'). This indicated that they serve as an annealing site for tRNA^{MET} for priming the synthesis of pre-genomic RNA molecules, as suggested previously for other members of the family *Caulimoviridae* (Schoelz, 2008).

Discussion

GVCV-VRU is a new isolate of GVCV that was discovered in a wild *V. rupestris* grapevine in Southwest Missouri. Its genome shares 91.5% identity at the nucleotide level with that of the original reference isolate GVCV-CHA. The most variable region between the two isolates is ORF II due to a 9 bp insert within GVCV-VRU. A separate study (Kovens et al.) showed both isolates to result in the characteristic vein clearing symptoms of GVCV, but also that GVCV-VRU infection produces necrotic flecks as the clearing veins of mature leaves progresses. The molecular basis for differences in symptom expression remains unknown although it is speculated that the 9 bp insert in the most variable ORF II may play a role in the formation of the necrotic flecks.

GVCV has not been reported in other viticultural areas of the USA where cultivars of *V. vinifera* are mainly grown, suggesting that it is endemic to the mid-western states and indigenous in native *Vitis* populations. The existence of a GVCV reservoir in native *Vitis* species in the forest and the potential for it to spill over into other wild *Vitis* species, as well as cultivated grapes, are a concern to the preservation of the declining wild *Vitis* populations and the prevention of viral diseases in commercial vineyards. It is

reasonable to hypothesize that GVCV isolates in native wild *Vitis* populations can spread to cultivated grapes after the cultivars of *V. vinifera* and their hybrids were introduced and planted in new vineyard sites. Therefore, we are currently investigating if GVCV-VRU can be found in cultivated *V. vinifera* and its hybrids in commercial vineyards. Alternatively, it is possible that GVCV also may spread from commercial vineyards to wild *Vitis* species. A comprehensive investigation involving large-scale sampling of commercial and native *Vitis* populations is required to better understand GVCV epidemiology and evolution.

In conclusion, we are continuing more research on GVCV, but there is still much more to be learned about GVCV. It is now understood that GVCV exists as a genetically diverse population that is capable of infecting both wild and cultivated vines. Also, symptoms are able to differ dependent upon the isolate, most likely due to whatever is coded for by ORF II, the least conserved region. However, we still don't know where or how this virus originated, and how it spreads or what vector transmitting it. Are there more isolates yet to be discovered and do their symptoms differ as well? What are the mechanisms that result in symptom expression, and of that, what do ORF I & II code for?

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