Metagenomic Analysis of Riesling Grapevine Reveals a Complex Virome Including Two New and Divergent Variants of *Grapevine leafroll-associated virus 3*

Huogen Xiao,¹ Caihong Li,¹ Maher Al Rwahnih,² Valerian Dolja,³ and Baozhong Meng^{1†}

¹Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada; ²Department of Plant Pathology, University of California, Davis, CA 95616, U.S.A.; and ³Department of Botany and Plant Pathology, Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR 97331, U.S.A.

Abstract

The virome of a major white wine grape of cultivar Riesling showing decline and leafroll disease symptoms was analyzed through high-throughput sequencing (HTS) using total RNAs as templates and the Illumina HiSeq 2500 platform. Analysis of HTS data revealed the presence of five viruses and three viroids in the infected vine. These viruses are *Grapevine leafroll-associated virus 1* (GLRaV-1) and GLRaV-3 (genus *Ampelovirus*, family *Closteroviridae*) and three viruses of the family *Betaflexiviridae* (namely, *Grapevine virus A* [GVA], *Grapevine virus B*, and *Grapevine rupestris stem pitting-associated virus* [GRSPaV]). We also show that multiple distinct strains of three viruses (GLRaV-3, GVA, and GRSPaV) were present in this diseased grapevine. The complete genomes of two novel and highly divergent isolates of GLRaV-3 were determined using the draft genomes derived from HTS data and two independent rapid amplification of cDNA ends (RACE) strategies to obtain sequences at both the 5' and the 3' termini of the viral genomes.

Questionable genome regions of both isolates were also verified through cloning of reverse transcription polymerase chain reaction products and Sanger sequencing. These two isolates are vastly divergent from all other isolates of GLRaV-3 whose genome sequences are available in GenBank. Isolate ON8415A has up to 76% nucleotide sequence identities to other isolates representing existing variant groups. We also revealed high degrees of variation in both length and sequence in the terminal untranslated regions (UTRs) of GLRaV-3 variants. The 5'-UTR of most GLRaV-3 isolates whose complete genomes have been sequenced contain tandem repeats of 65 nucleotides, a highly unusual feature rarely observed in (+)single-stranded RNA viruses. Mechanisms for the biogenesis of these tandem repeats and their function in virus replication and pathogenesis require investigation. Findings of this research add to the genetic diversity, evolutionary biology, and diagnostics of GLRaV-3 that afflicts the global grape wine industry.

The grape and wine industries constitute an important cornerstone of the economy of many countries. Diseases caused by viral pathogens are a major impeding factor for grape and wine production, as a result of a reduction in yield, quality of grapes, and wine products and the productive lifespan of vineyards (Mannini and Digiario 2017; Martelli 2017). Grapevine is host to a large number of evolutionary diverse viruses (Dolja et al. 2017). More than 80 distinct viral species that belong to 29 genera and 17 families have been identified to date (Martelli 2018). The greatest economic impact is caused by viruses involved in three major disease complexes: infectious degeneration and decline, grapevine leafroll disease (GLD), and rugose wood (RW) disease complex. More recently, two new diseases have been recognized: grapevine red blotch caused by *Grapevine red blotch virus* (GRBV) (Cieniewicz et al. 2017) and grapevine leaf mottling and deformation with *Grapevine Pinot gris virus* as the putative causal agent (Saldarelli et al. 2017).

Infectious degeneration and decline are caused by infection with multiple viruses from the genus *Nepovirus* (family *Secoviridae*), which are transmitted by nematodes (Digiario et al. 2017; Rowhani

et al. 2017). Diseases of the RW complex are putatively caused by several viruses of the family *Betaflexiviridae*, which include *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), and other related viruses of the genus *Vitivirus* (Minafra et al. 2017) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) of the genus *Foveavirus* (Meng and Rowhani 2017). The GLD complex is associated with several viruses that belong to three genera of the family *Closteroviridae*. *Grapevine leafroll-associated virus 1* (GLRaV-1) (Naidu, 2017), GLRaV-3 (Burger et al. 2017), and GLRaV-4 (Aboughanem-Sabanadzovic et al. 2017) are members of the genus *Ampelovirus* and are transmitted by multiple species of mealybugs (family *Pseudococcidae*) and scale insects (family *Coccidae*). GLRaV-2 is a member of the genus *Closterovirus* (Angelini et al. 2017), whereas GLRaV-7 is a member of the genus *Velarivirus* (Al Rwahnih et al. 2017). These two latter viruses are not known to be vector transmissible; in agricultural settings, they are transmitted through vegetative propagating materials and by grafting (Martelli et al. 2012; Naidu et al. 2014).

GLD is economically the most important viral disease of grapevine and GLRaV-3 is considered the predominant etiological agent of the disease (Burger et al. 2017; Maree et al. 2013). Earlier research revealed the existence of broad genetic diversity of GLRaV-3 (Bester et al. 2012; Chooi et al. 2013; Fei et al. 2013; Jooste et al. 2010; Maree et al. 2015). Through phylogenomic analysis of 13 available whole genomes and partial sequences, Maree et al. (2015) proposed the grouping of all GLRaV-3 isolates into seven variant groups, which were named by Roman numerals as groups I through VII. Complete genome sequences are available for viral isolates from groups I to III, VI, and VII. For variant group IV, only the 3'-terminal 4.6-kb sequences (corresponding to open reading frames [ORFs] 6 to 12) are available (Wang et al. 2011). For variant group V isolates, only the genomic region encoding the viral capsid protein (CP) was sequenced (Engel et al. 2008). These seven variant groups are further divided into three supergroups, designated as supergroups A through C (Maree et al. 2015). Supergroup A is the largest and comprises groups I to V.

†Corresponding author: B. Meng; bmeng@uoguelph.ca

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Supergroup B contains a single variant group (group VI) represented by three isolates (GH11, GH30, and CA7246) and two isolates from New Zealand (NZ-1 and NZ-1B) for which only the CP region has been sequenced (Chooi et al. 2013). Finally, supergroup C contains a single isolate (GH24), which was the only completely sequenced isolate at the time (Maree et al. 2015).

Major differences were observed among the different groups of viral variants. For instance, the small ORF2 is detected in isolates from groups I to V but is absent in isolates from groups VI and VII. In addition, the last two ORFs at the 3'-terminal region of the genome, ORF11 and ORF12, exhibit unusual levels of variability. This is especially the case for ORF11, as it is confidently predicted only in isolates of groups I to III. These observations raise the question of whether ORF11, and even ORF12, actually encodes a protein product (Maree et al. 2015). In light of the broad range of genetic diversities that have already been observed among GLRaV-3 isolates for which the genomes have been sequenced, it is likely that additional and genetically divergent viral variants of the virus still exist in nature that await to be identified.

First established in the 1970s, the Canadian grape and wine industry is relatively new. With the creation of the North American Free Trade Agreement, the Canadian grape industry has gone through major changes, with large proportions of *Vitis labrusca* and French-American hybrid wine cultivars being replaced by *V. vinifera* wine grapes. Ontario has remained the predominant producer of grape and wine, followed by British Columbia, Nova Scotia, and Quebec. However, the situation of grapevine viruses, viral diseases, and their economic impact on grape and wine production in Canada has remained largely unknown. An earlier survey for the distribution of a small number of viruses was conducted in the 1990s (MacKenzie et al. 1996), which revealed the presence of two viruses associated with GLD (GLRaV-1 and GLRaV-3) and two viruses involved in infectious degeneration (Grapevine fanleaf virus and Arabis mosaic virus). This situation changed drastically in recent years as a result of outbreaks of diseases indicative of viral infections in many vineyards. Since 2013, the negative effects of viruses and viral diseases on the health, productivity, and sustainability of the grape and wine industry in Canada started to be keenly recognized by various stakeholders, including growers, wineries, government agencies, and the research community. In order to understand the distribution and prevalence of viruses and their impact on the grape and wine industry, we have conducted province-wide surveys for major viruses. Fourteen viruses were detected in commercial vineyards, with GLRaV-3, GRBV, GPGV, and GRSPaV being the most prevalent (Xiao et al. 2018). Two recent surveys were conducted in British Columbia on the distribution and genetic diversity of GRBV and four viruses associated with GLD (Poojari et al. 2017a, 2017b).

During sample collection and visitations to various vineyards, we have witnessed, first hand, severe losses and even total crop failure in numerous vineyards. One of the most affected sites was an organic cultivar Riesling vineyard block located in the Niagara Peninsula. This vineyard was established in the early 1980s. The owner of this vineyard had been experiencing substantial economic losses owing to a pronounced reduction in both the yield and quality of grapes. At the grower's request, we visited this vineyard in late fall of 2014. The Riesling vines exhibited severe decline, stunted growth, and only a few clusters of grapes contrary to what would be expected of a normal vineyard of this age. We also observed yellowing and downward rolling of leaves, typical of GLD.

To identify the viruses associated with this declining vineyard block, we performed a global analysis of the virome in a grapevine representing this vineyard block using the Illumina HiSeq 2500 platform and total RNAs. Five species of viruses and three viroids were identified. These included two viruses involved in GLD, as expected based on the disease symptoms, and three viruses that are associated with the RW disease complex. Furthermore, multiple genetic variants were detected for three of these viruses. Importantly, two of the sequenced GLRaV-3 variants were only distantly related to previously characterized variants of this virus.

Materials and Methods

Plant materials. Dormant cuttings were collected from a vine of the diseased Riesling in November 2014 from an organic vineyard in Niagara, Southern Ontario, Canada. We designated this vine as ON8415.

Isolation of total RNAs. Total RNAs were isolated from phloem scrapings of cuttings by using our modified method based on the Spectrum Plant Total RNA Kit (Sigma) (Xiao et al. 2015). The quality and concentration of the RNA preparations was assessed with a NanoDrop spectrophotometer (ND-1000; Thermo Scientific) at wavelengths of 230, 260, and 280 nm. The nucleic acid preparations were also evaluated with microfluidic electrophoresis chips using the BioAnalyzer system (Agilent Technologies).

High-throughput sequencing. The total RNAs were subjected to the removal of ribosomal RNAs (rRNAs) and the rRNA-depleted RNA samples were used for preparation of a cDNA library using the Illumina TruSeq RNA Sample Prep Kit. Sequencing was carried out on an Illumina HiSeq 2500 sequencer generating 51-bp single-end reads at the Donnelly Sequencing Centre, University of Toronto. The high-throughput sequencing (HTS) data set was first analyzed in the iPlant Discovery Environment. Initially, sequence reads from the cDNA library were mapped to the reference genome of *V. vinifera* (PRJEA18785) using TOPHAT2-SE to enrich the sequence reads related to viruses and viroids. Sequence reads not mapped to the grapevine reference genome were mapped using the Basic Local Alignment Search Tool (BLAST)-Like Alignment Tool available in iPlant to reference sequences corresponding to viruses and viroids. These nongrape sequence reads were also subjected to de novo assembly into contigs using CLC Genomics Workbench (Qiagen). Resulting contigs were subsequently used as queries to identify matching sequences using BLAST against complete reference sequences of viruses and viroids (<http://www.ncbi.nlm.nih.gov/genome/viruses/>) to identify viruses and viroids that were present in the sample. To obtain the complete or partial genome sequences of the viruses and viral variants detected, de novo assembled viral contigs were compared manually with individual viral genome sequences available in GenBank, which resulted in the assembly of draft genomes of various variants. Reference sequences used for comparison and for the identification of viral variants are provided in Table 1.

Confirmation of novel GLRaV-3 variants by reverse transcription polymerase chain reaction and Sanger sequencing. Near full-length genome sequences of two new variants of GLRaV-3, designated as ON8415A and ON8415B, were assembled using sequence data from HTS. For the purpose of verification of the draft genome sequences, primers were designed to amplify regions of each new variant that displayed significant deviations compared with other isolates that have been published Supplementary Table S1. Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously (Xiao et al. 2015) using primers designed based on the draft genome sequence of each novel isolate. Resulting PCR products were analyzed on 1% agarose gel and amplicons of expected sizes were cloned into pGEM-T Easy Vector (Promega). Resulting positive clones were sequenced with M13 forward and reverse primers using an ABI 3730 capillary sequencer at the Genomics Facility of the Advanced Analysis Centre, University of Guelph.

Determination of the 5' and 3' termini of the genome of new isolates. The FirstChoice RNA ligase mediated (RLM)-RACE Kit (Thermo Fisher Scientific) was used to determine the 5' and 3' termini of the genome of isolates ON8415A and ON8415B. Two reverse primers (shared by ON8415A and ON8415B) and one forward primer for each isolate were designed for 5' RLM-RACE, whereas two forward primers for each isolate and one reverse primer (shared by both isolates) were designed for 3'-RACE. Because the genome of GLRaV-3 lacks a poly(A) tail, poly(A)-tailing was first performed on total RNAs isolated from the infected vine with yeast poly(A) polymerase (USB). The 5'- and 3'-RACE was performed following the vendor's instructions. To further verify the 5'-terminal nucleotide of the genomes of these two new isolates, an independent

RACE was conducted. Briefly, cDNA was prepared with specific primer 8415AB-5outR plus random primers using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Poly(C)-tailing of the resulting cDNA was done using terminal deoxynucleotidyl transferase (New England BioLabs). This was followed by PCR amplification with primers 8415AB-5outR and dG-14 for the first-round PCR and 8415AB-5inR and dG-14 for the nested PCR. The resulting RT-PCR products were analyzed through electrophoresis on 1% agarose gel and the amplicons of expected sizes were cloned into pGEM-T Easy Vector as described earlier. Positive clones were first screened by colony PCR with virus-specific primers, followed by plasmid isolation and sequencing with M13 forward and reverse primers.

Phylogenetic analyses. Multiple sequence alignments and phylogenetic analyses were performed by using MEGA7 available online. The percentage identity and the phylogenetic tree were obtained through aligning the nucleotide or amino acid sequences using ClustalW. Isolates of GLRaV-3 used in the phylogenetic analyses were as follows: 623 (GQ352632), GH24 (KM058745), Cl-766 (EU344893), GP18 (EU259806), WA-MR (GU983863), PL-20 (GQ352633), 621 (GQ352631), NY1 (AF037268), LN (JQ423939), CA7246 (JQ796828), GH30 (JQ655296), GH11 (JQ655295), TRAJ-BR (KX756669), TC-BR (KX756668), ISAB-BR (KX701860), 3138-07 (JX559645), an isolate from Israel (KJ174518), and genome sequences of several other isolates that became available in GenBank, which include Pro95 (KY707824), Trc138 (KY764333), Trc139 (KY764332), Rod96 (KY707825), NdA121 (KY707826) (Maher et al., unpublished data), and ID45 (MH796136; Thompson et al. 2019). GLRaV-1 isolate WA-CH (KU674796) was included as an outgroup. For further phylogenetic analyses of UTRs and select ORFs, only a subset of isolates representing genetic variant groups was included. Several isolates were excluded from further phylogenetic analyses either because these isolates were not completely sequenced (isolates Cl-766 and NY1) or unverified sequences are available (isolate 139).

Results

A single vine contains multiple viruses and viral variants. Deep sequencing was used to identify the viruses and viroids in a Riesling vine exhibiting leafroll and general decline symptoms. Total RNA was isolated from cane phloem tissue and used for generation of a cDNA library, followed by single-end sequencing on the HiSeq 2500 platform. A total of 73,385,886 sequence reads were obtained from a ribo-depleted cDNA library derived from the sample designated ON8415 Supplementary Table S2. Among them, 23,026,032 reads were not mapped to the *V. vinifera* genome. These reads were mapped against the GenBank database of viruses and viroids (<http://www.ncbi.nlm.nih.gov/genome/viruses/>). A total of 5,930,512 reads (25.8%) matched sequences of the following five viruses and three viroids: GLRaV-1, GLRaV-3, GRSPaV, GVA, GVB, Grapevine

yellow speckle viroid 1 (GYSVd1), GYSVd2, and Hop stunt viroid. The total number of virus and viroid reads accounted for 8.08% of the total sequence reads, indicative of significant levels of virus and viroid accumulation in this Riesling vine. By far, the most abundant virus was GLRaV-3 (84.93%), followed by GVB (11.49%) (Fig. 1).

Resulting contigs were investigated using BLAST against complete reference sequences of viruses, followed by manual comparisons with known genome sequences of the respective viruses and by assembly into draft genomes of individual virus variants (Table 1). Near complete genome sequences of three distinct variants of GLRaV-3 were assembled from the sequence reads derived from this sample (Table 1). Contigs 5, 8, and 12 were assembled into the draft genome of the first variant, which had 99% sequence identity and 96.6% genome coverage with isolate 623 (GQ352632; Jooste et al. 2010); the second was assembled from contigs 7 and 86 and consisted of 18,320 nt. Designated ON8415A, this draft genome had low nucleotide sequence identity of 76.2% to GH24 (KM058745; Maree et al. 2015), with a 99.1% genome coverage.

Fig. 1. Distribution of viruses and viroids identified in the cDNA library derived from sample ON8415. The abundance of sequence reads for the viruses is expressed as the percentage of sequence reads for each virus or viroid over the total number of virus/viroid reads. GYSVd = Grapevine yellow speckle viroid, HSVD = Hop stunt viroid, GRSPaV = Grapevine rupestris stem pitting-associated virus, GLRaV = Grapevine leafroll-associated virus, GVA = Grapevine virus A, and GVB = Grapevine virus B.

Table 1. Summary of assembled sequence contigs from sample ON8415 and their mapping to viruses and viral variants^a

Virus ^b	GenBank accession no.	Reference isolate	Sequence identity (%)	No. of contigs	Genome coverage (%)	New isolates with full genome sequence (GenBank accession no.)
GRSPaV	KT948710.1	VF1	94–96	4	97.3	
	HE591388.1	SY	96–97	3	96.1	
	FR691076.1	MG	98	2	96.3	
	JQ922417.1	GG	97–98	5	17.8	
GVA	KC962564.1	I327-5	83	5	96.1	
	DQ855084.2	GTG11-1	82	9	77.9	
	JX559641.1	3138-03	79	1	93.3	
GVB	X75448.1	Italy	97	1	100	ON8415 (KY426923)
GLRaV-1	KU674796	WA-CH	99	2	99.9	
GLRaV-3	GQ352632.1	623	99	3	96.6	
	KM058745.1	GH24	76.2	2	99.1	ON8415A (KY073323)
	GQ352633	PL20	67.1	3	99.3	ON8415B (KY073324)

^a De novo assembled viral sequence contigs from sequence reads derived from the cDNA library derived from sample ON8415 were manually compared with the known viral genome sequences followed by assembly into various virus variant sequences.

^b GRSPaV = Grapevine rupestris stem pitting-associated virus, GVA = Grapevine virus A, GVB = Grapevine virus B, and GLRaV = Grapevine leafroll-associated virus.

The third variant, which is designated here as ON8415B, was assembled from contigs 4, 16, and 22, consisted of 18,373 nt, and had only 67.1% sequence identity on average compared with isolate PL-20 (Jooste et al. 2010), with a 99.3% genome coverage (Table 1).

In addition, three new and divergent variants of GVA were also detected. Variant 1 had 83% sequence identity with I327-5 (KC962564; Goszczynski 2014); variant 2 was 82% identical to isolate GTG11-1 (DQ855084; Goszczynski 2007); and variant 3 had 79% identity to isolate 3138-03 (JX559641; Rott and Belton, *unpublished data*). In contrast, only a single variant of GVB was detected. The complete genome of GVB was assembled from sequence reads from this sample (GenBank accession no. KY426923), which had 97% sequence identity with X75448.1 from Italy (Saldarelli et al. 1996) (Table 1). Furthermore, four variants of GRSPaV as well as a single GLRaV-1 variant were also detected (Table 1).

Completion of genome sequences of two new and divergent GLRaV-3 isolates. Based on assembly of sequence reads derived from HTS, the draft genome sequences for both ON8415A (18,250 nt) and ON8415B (18,373 nt) were obtained. Alignment with the complete genome of isolate GH24 suggested that both draft genome sequences were incomplete at each end of the genome, which also exhibited apparent deletions and insertions in both terminal regions as well as regions of low confidence in sequence quality throughout the genome. Therefore, we set out to complete and validate the draft genome sequences of these two new GLRaV-3 isolates.

To obtain the sequence corresponding to the 5' termini of ON8415A and ON8415B, we performed RACE using two independent RACE approaches. First, we used the RLM-RACE system (Thermo Fisher Scientific) and two reverse primers conserved for both variants together with a primer targeting the RNA adaptor provided in the commercial kit. After RT-PCR and cloning, six clones were obtained, two of which (310-A1 and 310-A2) were specific for ON8415A and four for ON8415B (310-A4, 310-A5, 310-A7, and 310-A8). The ON8415A-related clones were nearly identical to each other and extended the genome sequence by 220 nt beyond the initial draft genome. It is important to note that the draft genome sequence was missing 70 nt (nucleotide positions 572 to 641 based on the final complete genome). Among the four ON8415B-related clones, three had apparently nonviral sequences beyond the matching region. BLAST searches demonstrated that these sequences were derived from the cytochrome P450 gene of grapevine. The remaining clone 310A-7 matched the 5'-terminal sequence of the draft genome and extended it by 28 nt.

Because the available RLM-RACE kit did not allow us to distinguish whether the very 5'-terminal nucleotide of the genome is a single or double "A," we used the terminal deoxynucleotidyl transferase for C-tailing of the first-strand cDNA, followed by PCR with oligo (dG) primer and virus-specific reverse primers. This RACE approach allowed us to confidently identify "A" as the 5'-terminal nucleotide for both ON8415A and ON8415B.

To obtain the 3'-terminal sequence for each of the new GLRaV-3 variants, we performed A-tailing of total RNAs, followed by RT-PCR with oligo(dT) primer and virus-specific forward primers and cloning of the resulting products. This approach allowed us to extend the draft genome sequences of ON8415A and ON8415B by 34 and 162 nt, respectively. It was determined that both genomes possessed a "C" as the last nucleotide, similar to most of the other known GLRaV-3 isolates available from GenBank (Fig. 2).

To confirm the sequences corresponding to the internal regions of uncertainty, we conducted RT-PCR using primers designed to flank each uncertain region, followed by cloning of PCR products into a TA vector and sequencing. Specifically, primer pair LR3-8415A-8882F and LR3-8415A-10433R was used to amplify the genomic region containing the internal questionable region between nucleotide positions 9,200 and 10,450. As a result, two clones (289-A2 and 289-A3) were selected for sequencing, with both clones being 99.8% identical and matching perfectly to the draft genome. A similar strategy was used to verify the uncertain regions in the draft genome of ON8415B. Specifically, we designed a second pair of primers (LR3-8415B-16223F and LR3-8415B-17153R) and used them in

RT-PCR to amplify the region of uncertainty located at nucleotide positions 16,252 to 17,153. As a result, we obtained and sequenced two clones: 249-B2 and 249-B3. Sequences of both clones were 99.6% identical to each other and had a nearly perfect match to the draft genome. Similarly, to verify the uncertain region located between nucleotide positions 4,200 and 5,700, primers LR3-RLB4196F and LR3-RLB5703R were designed and used in RT-PCR, and the amplification products were cloned. Because both clones had inserts of similar size based on restriction digestion, we sequenced one of them (T-PCR-C4). The sequence of this clone was identical to the draft genome sequence except for a single mismatch.

Upon compilation of the sequences derived from HTS, from the 5'- and 3'-RACE, and from RT-PCR targeting internal regions, the complete genome sequences for both ON8415A and ON8415B were obtained. The genome of ON8415A (KY073323) is 18,573 nt in length, contains a 5'-UTR of 737 nt, a 3'-UTR of 303 nt, and 12 ORFs lacking ORF2. The genome of ON8415B (KY073324) is 18,564 nt long, with a shorter 5'-UTR of 672 nt, a longer 3'-UTR of 350 nt, and 13 ORFs including ORF2. Overall, the two isolates differ from each other by 33.1% in genome sequence.

ON8415B is a unique and highly divergent isolate of GLRaV-3. At the time this work was being completed, seven phylogroups of GLRaV-3 variants had been recognized (Maree et al. 2015). These groups were further classified into three supergroups designated supergroup A (groups I to V), supergroup B (group VI), and supergroup C (group VII, GH24). For unknown reasons, the partial sequences from Portugal representing group VIII were removed from GenBank; hence, group VIII and supergroup D are obsolete. As this manuscript was being reviewed for publication, genome sequences of several isolates became available in GenBank, including five from California (Pro95 [KY707824], Trc138 [KY764333], Trc139 [KY764332], Rod96 [KY707825], and NdA121 [KY707826]) and one from Idaho (ID45 [MH796136]). Thompson et al. (2019) proposed group IX as a new group to contain four highly similar isolates: ID45, Pro95, Trc138, and Rod96.

The nucleotide sequence identities of isolate ON8415B to other GLRaV-3 genomes were very low and fell in the range of 66.8 to 72.4%, which are much lower compared with identity values of ~88 to 91% between variants across groups I, II, III, and VI. Based on these results, we conclude that ON8415B is a highly divergent isolate that appears to be a sister variant more closely related to group IX (Fig. 3A). Similar to isolate PL-20 (group III), the 5'-UTR of ON8415B is 672 nt in length (Fig. 4), shorter than most other isolates. However, the sequence identity of the 5'-UTR between ON8415B and other isolates is very low, ranging from 45.8 to 56.1%. In contrast, it has the longest 3'-UTR (350 nt) of any completely sequenced GLRaV-3 variants (Fig. 2). The sequence identities between the 3'-UTR of ON8415B and those of other variants range from 69.7 to 86.2% (Table 2). The identity levels for ORF1b, ORF4, and ORF6 of ON8415B relative to other variants are 75.6 to 77.6, 72.9 to 79.4, and 75.0 to 82.0%, respectively, in nucleotide sequence (Table 2); the corresponding amino acid identities are 87.2 to 89.1, 84.2 to 90.9, and 88.5 to 96.2% (Table 3). Considering the large divergence of this isolate compared with members of group IX, it seems reasonable to suggest that ON8415B may represent a new genetic variant group of GLRaV-3.

We also noted that ON8415B appeared to be more closely related to groups I to III than to groups VI, VII, and IX when the 5'-UTRs were compared. In contrast, it appears more closely related to groups VI and VII than to groups I to III when the other genomic regions were compared (*data not shown*). This incongruence over different regions of the genome is also reflected in the phylogenetic trees where ON8415B is positioned beside the clade of groups I to III for ORF1a (*data not shown*), ORF4 (SF1B), and ORF6 (Fig. 3B), whereas it is positioned between groups VI and VII in the tree for ORF1b (SF1A). This suggests that recombination events might have taken place between different viral variant groups in the past, leading to the emergence of isolate ON8415B.

ON8415A is distantly related to group VII variants of GLRaV-3. Sequence analysis of multiple regions across the genome revealed

encoding RdRP (ORF1b), Hsp70h (ORF4), and major capsid protein (ORF6) were 76.6 to 84.4, 72.8 to 79.6, and 74.8 to 82.7% identical compared with other GLRaV-3 isolates, respectively (corresponding amino acid identities were 88.2 to 93.7, 82.7 to 88.9, and 89.8 to 96.2%) (Tables 4 and 5). Sequence differences between ON8415A and other variant groups were even greater when the remaining nine ORFs were compared, which are generally less conserved among members of the family *Closteroviridae* (Tables 4 and 5) (Dolja et al. 2006). The nucleotide sequence identities of variants among groups I, II, and III are greater than 87.6% in these three ORFs, which is considerably higher than that between ON8415A and groups I to III (with the highest identity value of 82.7%, see above). Furthermore, the identities of members of the highly variable group VI are greater than 90.8%.

The 5'-UTRs of GLRaV-3 variants including ON8415A and ON8415B are highly variable and contain tandem repeats. The 5'-UTR of GLRaV-3 exhibits unusual features that are not observed in other viruses outside the genus *Ampelovirus*. First, the 5'-UTRs of GLRaV-3 isolates are unusually long and vary in length considerably among the different variants, ranging from 642 nt (for GH30) (Bester et al. 2012) to 802 nt (for LN) (Fei et al. 2013). Isolates ON8415B and PL-20 both have a 5'-UTR of 672 nt (Fig. 4). Second, all of the variants for which complete genomes have been sequenced thus far contain tandem repeats of 65 nt in the 5'-UTR, albeit the levels of sequence identity between these repeats vary from virus to virus. In addition, the copy numbers of repeats also vary, ranging from 2 to 4 (Fig. 5). It is interesting that the new isolate ON8415A contains four copies of repeats with sequence identities of 69.2 to 83.1% between them (Fig. 5). Third, the 5'-UTRs of different variants exhibit

extensive sequence variation. For example, the 5'-UTR of isolate ON8415A shares sequence identities from 47.9 (to PL-20) to 60.6% (to ID45). Similarly, the 5'-UTR of isolate ON8415B has the lowest identity of 45.8% (with PL-20) to the highest 56.1% (with ID45) (Table 2). It is worth noting that in spite of these variations in both sequence and length of the 5'-UTRs, the first ~40 nt of all completely sequenced genomes are nearly invariable, suggesting critical functions of this sequence segment in viral genome replication (Fig. 4).

The 3'-UTRs of GLRaV-3 isolates vary in length and nucleotide sequence. Similar to the 5'-UTRs, a high degree of variability is observed in the 3'-UTRs of different GLRaV-3 variants. Their size ranges from 259 nt for GH24 to 350 nt for ON8415B, with those from groups I to III all being 277 nt (Fig. 2). The 3'-UTR of ON8415A is 303 nt in length. Second, they also exhibit differences in sequence, with identities ranging from 69.7 to more than 90% among variants. The identities between the 3'-UTR of ON8415A and those of other variants range from 69.7 (with ON8415B) to 90% (with GH24). The identities between the 3'-UTR of ON8415B and those from other variants range from the lowest 69.7% (with ON8415A) to the highest 86.2% (with WA-MR). A majority of these differences are located in the 5'-proximal one-third of the 3'-UTR. In contrast, the 3'-proximal two-thirds of the 3'-UTR of GLRaV-3 isolates are more conserved and contain several invariable stretches of sequences ranging in size from 16 to 22 nt (Fig. 2).

Discussion

In this study, we further demonstrate the utility of HTS for the global and unbiased identification of viruses from crop plants. This technology is especially attractive for the asexually propagated, perennial woody plants such as grapevine (Al Rwahnih et al. 2009, 2012a, 2012b; Coetzee et al. 2010; Fajardo et al. 2017; Giampetruzzi et al. 2012; Glasa et al. 2014, 2017; Maliogka et al. 2015; Poojari et al. 2013; Zhang et al. 2011). Together with other recent HTS studies (Al Rwahnih et al. 2009; Coetzee et al. 2010; Pantaleo et al. 2010; Zhang et al. 2011), our work revealed a highly complex nature of

Fig. 3. Phylogenetic analyses of *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates representing sequence variant groups. Phylogenetic trees were generated based on **A**, complete or near complete genomes and **B**, open reading frame 6 (ORF6). One representative isolate from each variant group was used for the construction of phylogenetic trees. The GenBank accession number and name are provided for each isolate. Grouping of isolates into viral variant groups and supergroups is also provided. Phylogenetic trees shown were generated using the maximum likelihood method with 1,000 replications of bootstrapping. Trees with similar topology were obtained using the Neighbor Joining method.

Fig. 3. (Continued)

viral infections in grapevine. Three types of nucleic acids have been used as templates in HTS for the identification of grapevine viruses, which include double-stranded RNA (Al Rwahnih et al. 2009, 2012b, 2013; Coetzee et al. 2010), small RNA (Fajardo et al. 2017; Giampetruzzi et al. 2012; Glasa et al. 2014; Maliogka et al. 2015; Navarro et al. 2009; Pantaleo et al. 2010; Velasco et al. 2014; Zhang et al. 2011), and total RNAs (Al Rwahnih et al. 2009; Beuve et al. 2015; Glasa et al. 2017; Jo et al. 2015). In this study, we have

demonstrated that total RNAs are as effective, and perhaps even better, for the global identification and genome assembly of viruses, including both RNA viruses (this study) and DNA virus (*our unpublished data*). We predict that total RNAs will become the method of choice in future studies for the identification and genome sequencing of viruses in grapevine and other plant species.

One of the most important outcomes of this study is the discovery and sequencing of the complete genomes of two new and highly

Fig. 4. Multiple alignment of 5'-untranslated regions (UTRs) of representative isolates for which the complete genomes have been sequenced. GenBank accession numbers, isolate names, and nucleotide positions are provided for the isolates included in the alignment. Note that the first 40 nucleotides at the 5' termini of genomes are highly conserved among isolates.

divergent variants of GLRaV-3 (ON8415A and ON8415B), which are only distantly related to other known virus isolates (Fig. 3; SF1). Prior to this work, seven phylogenetic groups of GLRaV-3 variants had been recognized by the international grapevine virology community (Burger et al. 2017; Maree et al. 2015). As this manuscript was in the process of peer review, group IX was proposed to represent several isolates from the United States (Thompson et al. 2019). Based on the phylogenetic analyses using either full genome sequence or several individual ORFs, it is evident that isolate ON8415A is sister to the cluster of variant group VII represented by GH24 (Fig. 3; Supplementary Fig. S1). On the other hand, the grouping of ON8415B cannot be readily resolved at the present time. ON8415B can be regarded to represent a new variant group of GLRaV-3 based on two reasons. First, ON8415B is only 72.4% identical or lower in genome sequence compared with members of group IX, which is much lower than intragroup sequence identity values for any other variant groups. Second, additional partial sequences of HSP70h that are closely related to ON8415B (with sequence

identities of ~93%) exist in GenBank, suggesting that similar variants are present in other regions of the world. Third, a preliminary survey and cloning work has confirmed the presence of nearly identical sequences in two other *V. vinifera* cultivars—namely, Chardonnay and Gewürztraminer (our unpublished data). Specific designation of this new viral variant group needs to be determined.

It is evident that GLRaV-3, similar to Citrus tristeza virus (a member of the genus *Closterovirus* that infects another woody perennial), exists as a large family of highly divergent viral variants. It is most likely that additional divergent isolates may be uncovered from grapevines that are of genotypes other than *V. vinifera* or that have been autochthonous varieties in less explored regions of the world such as Asia and the Middle East. Our preliminary HTS data from additional infected grapevine samples seem to support this hypothesis. To unify the classification system for grouping both known and additional divergent variant groups of GLRaV-3 to be identified in the future, and to avoid confusions that may arise in the literature, we call upon the international grapevine virology community to

Table 2. Comparison of nucleotide sequences of whole genome and genomic regions between isolate ON8415B and select reference isolates representing known variant groups of *Grapevine leafroll-associated virus 3* (GLRaV-3)^a

Isolate	Full	5'-UTR	ORF1a	ORF1b	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7	ORF8	ORF9	ORF10	ORF11	ORF12	3'-UTR
WA-MR	72	45.9	68.2	75.7	78.8	77.5	78.2	76.1	82.0	76.7	80.3	70.8	69.4	52.3	70.3	86.2
623	72.1	46.5	68.3	75.9	75.0	76.1	78.5	76.0	81.4	77.3	79.6	70.8	70.2	55.0	70.9	85.8
PL-20	72	45.8	68.0	75.6	74.4	74.6	78.1	76.4	80.9	78.1	80.3	71.2	71.3	55.0	70.3	85.5
CA7246	67.8	50.4	67.3	77.1	NA	73.2	72.9	69.1	80.5	72.0	73.3	62.5	63.1	43.1	60.6	73.1
GH11	67.8	51.9	67.5	77.6	NA	76.1	73.7	69.2	80.5	72.0	74.2	61.0	66.3	39.4	64.2	74.1
8415A	66.9	54.8	65.8	76.6	NA	72.5	73.8	69.3	75.5	67.5	70.6	56.5	57.8	45.0	66.7	69.7
GH24	66.8	51.1	65.7	76.1	NA	75.4	73.3	69.6	75.0	67.7	68.6	56.3	60.7	44.0	62.4	74.3
ID45	72.4	56.1	68.4	76.9	NA	80.4	79.4	77.8	81.4	77.7	78.5	72.5	74.1	NA	68.5	79.7

^a The whole genome and nucleotide sequences of genomic regions, open reading frames (ORFs), and terminal untranslated regions (UTRs) of isolate ON8415B were compared with their corresponding sequences of other GLRaV-3 reference isolates for which complete genome sequences are available in GenBank. Nucleotide identities are given as percentage values. Isolates CA7246, GH11, GH30, ON8415A, and GH24 lack ORF2; therefore, the comparisons are not available (NA).

Table 3. Comparison of amino acid sequences of open reading frames (ORFs) between isolate ON8415B and select reference isolates whose complete genome sequences are available^a

Isolate	ORF1a	ORF1b	ORF2	ORF3	ORF4	ORF5	ORF6	ORF7	ORF8	ORF9	ORF10	ORF11	ORF12
WA-MR	71.3	88.7	64.7	80.0	90.2	81.6	93.3	84.7	87.0	69.5	70.9	30.6	75.9
623	71.8	86.9	54.9	82.2	90.9	81.2	92.0	85.5	87.0	70.1	68.7	33.3	75.9
PL-20	71.2	88.2	58.8	77.8	89.8	80.7	92.0	85.5	82.2	69.5	77.1	36.1	74.1
CA7246	71.4	89.1	NA	80.0	85.4	76.0	93.6	78.8	77.8	59.9	65.9	25.0	63.0
GH11	71.7	88.9	NA	84.4	85.1	75.2	96.2	78.8	78.4	56.5	68.2	22.2	64.8
8415A	69.3	88.2	NA	75.6	84.2	75.6	90.7	75.3	73.0	51.9	51.4	25.0	59.3
GH24	69.4	87.2	NA	75.6	85.2	75.6	88.5	75.3	69.7	50.6	55.9	25.0	66.7
ID45	71.2	88.1		84.8	89.3	83.9	92.4	84.1	80.1	66.9	75.0	NA	72.7

^a The amino acid sequence of each ORF of isolate ON8415B and those of other isolates whose complete genomes are available in GenBank were compared using the ClustalW method. Amino acid identities are given as a percentage. Isolates CA7246, GH11, GH30, ON8415A, and GH24 lack ORF2; therefore, the comparisons are not available (NA).

Table 4. Comparison of nucleotide sequences of whole genome and genomic regions between isolate ON8415A and select reference isolates representing known variant groups of *Grapevine leafroll-associated virus 3* (GLRaV-3)^a

Isolate	Full	5'-UTR	ORF1a	ORF1b	ORF3	ORF4	ORF5	ORF6	ORF7	ORF8	ORF9	ORF10	ORF11	ORF12	3'-UTR
WA-MR	66.9	49.5	65.2	79.1	69.5	74.2	69.8	74.8	69.0	69.2	57.6	55.2	38.5	62.1	74.0
623	66.8	49.7	65.4	78.8	71.0	74.0	70.0	75.0	68.9	70.4	55.4	54.8	43.1	64.4	75.1
PL-20	67.0	47.9	65.4	79.4	67.4	73.0	70.1	76.5	68.8	68.8	57.3	56.1	42.2	65.0	75.1
CA7246	67.2	57.0	67.2	79.8	66.7	73.1	69.4	76.1	70.6	67.4	53.5	57.2	54.1	61.8	79.5
GH11	67.1	56.2	67.5	79.9	68.1	72.8	68.7	77.6	69.3	67.4	54.6	57.6	48.6	64.2	81.7
8415B	66.9	54.8	65.8	76.6	72.5	73.8	69.3	75.5	67.5	70.6	56.5	57.8	45.0	66.7	69.7
GH24	76.2	60.1	75.2	84.4	72.5	79.6	76.4	82.7	76.2	78.3	75.1	75.0	64.9	70.0	90.0
ID45	67.4	60.6	67.2	77.0	71.7	73.5	69.4	76.0	66.7	67.4	58.9	56.9	NA	63.8	73.6

^a The whole genome and nucleotide sequences of genomic regions, open reading frames (ORFs), and terminal untranslated regions (UTRs) of isolate ON8415A were compared with their corresponding sequences of other GLRaV-3 reference isolates for which complete genome sequences are available in GenBank. Nucleotide sequence identities are given as percentage values. Isolate ON8415A lacks ORF2; therefore, the comparisons are not available (NA).

assess and refine the classification system of GLRaV-3 variants in order to reflect these additional new variants.

Although the first ~40 nt of all sequenced GLRaV-3 genomes are highly conserved, a high degree of variability exists among the different isolates for which the complete genomes have been sequenced. These variations are most profound in the noncoding regions of the viral genome. For example, the 5'-UTR exhibits large variability in both length and sequence. They range from 642 to 802 nt, with a majority of isolates having a 5'-UTR of 737 nt as first shown by Maree et al. (2008). Moreover, tandem repeats of 65 nt were first reported in the 5'-UTR of GLRaV-3 isolates from Washington State (Jarugula et al. 2010). Here, we expand on this initial observation and reveal that these 65-nt-long tandem repeats exist in all isolates for which

the genomes have been sequenced, albeit with different degrees of diversification between repeats. Similar repeats were also detected in the 5'-UTR of two other members of the genus *Ampelovirus*, GLRaV-1 (Donda et al. 2017) and GLRaV-13 (*data not shown*). GLRaV-13 contains seven copies of such tandem repeats, with 65 to 86% sequence identities among them. Interestingly, these 65-nt repeats seem to be a hallmark for only grapevine-infecting members of the subgroup I of the genus *Ampelovirus*. In other words, similar repeats are not detected in GLRaV-4 and its strains, which belong to subgroup II of the genus *Ampelovirus*, or in any nongrapevine-infecting members of the genus. The presence of tandem repeats in GLRaV-3 and related viruses GLRaV-1 and GLRaV-13 is rather bizarre, as such a phenomenon is generally not seen in single-stranded,

Table 5. Comparison of amino acid sequences of open reading frames (ORFs) between isolate ON8415A and select reference isolates whose complete genome sequences are available^a

Isolate	ORF1a	ORF1b	ORF3	ORF4	ORF5	ORF6	ORF7	ORF8	ORF9	ORF10	ORF11	ORF12
WA-MR	68.9	91.3	71.1	85.8	75.2	90.7	74.8	70.8	53.4	47.5	19.4	58.2
623	69.2	89.6	73.3	86.2	75.4	89.8	75.3	72.4	51.1	48.6	25.0	56.9
PL-20	69.2	90.2	71.1	86.0	76.0	89.8	74.2	70.3	51.7	50.3	25.0	58.6
CA7246	70.9	91.0	75.6	83.1	74.3	90.7	76.1	69.2	46.0	48.0	44.4	53.7
GH11	71.1	90.8	75.6	82.7	72.7	91.4	74.0	67.6	45.5	51.4	36.1	48.1
8415B	69.3	88.2	75.6	84.2	75.6	90.7	75.3	73.0	51.7	51.4	25.0	59.3
GH24	80.2	93.7	80.0	88.9	83.6	96.2	81.6	84.9	76.1	78.2	55.6	66.1
ID45	70.2	88.9	78.3	84.2	76.0	88.5	73.4	69.4	52.5	47.2	NA	59.3

^a The amino acid sequences of each ORF of isolate ON8415A and those of the other *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates are compared using the ClustalW method. Amino acid identities are given as percentage values. Isolate ON8415A lacks ORF2; therefore, no comparisons were made for ORF2 (NA).

A

Variants	Variant groups	Position of repeats in genome	Size of repeats	Copy number of repeats	Percent identity among repeats
WA-MR	I	186-321	65	2	92
623	II	84-217	65	2	72
LN	III	149-287	65	2	94
PL-20	III	116-286	65	2.6	90
GH11	VI	186-321	65	2	90
CA7246	VI	237-365	65	2	92
GH24	VII	121-257	65	2	74
ON8415A	TBD	185-444	65	4	81
ON8415B	TBD	121-321	65	3	60
ID45	IX	113-388	65	4	75

B Consensus #1 TTTCTT...T...A...AAA.A...AA.TTT.T...T...T.TTGTG.GTG...GT.G...TT...G.

8415A_repeat1 TTTCTTAGTATTAAGAAAAAGAAATTTTCTCTTCTTTTGTGTTGTGTTGTTCTTTTGGGT 65
8415A_repeat2 TTTCTTAATATAGTAAAAAGAAATTTTCTTTCTTCTTTTGTGTTGTGTTGTTCTTTTAGGC 65
8415A_repeat3 TTTCTTTATTTAGTAAAAAGAAATTTTCTTTATTTCTTGTGTTGTGTTGTTCTTTTAGGC 65
8415A_repeat4 TTTCTTTATAAGTAAAAAGAAATTTTCTTTCTTCTTTTGTGTTGTTGTTGTTCTTTTAACT 65

C Consensus #1 .TT.CTAA.....A..A.AAAA.TA.TC...TTTT...T...TT...T.TG...T...AG..

ON8415B_repeat1 TTTCTAAAACTCTTAAAAAGAAAAATAGTCCCTTTTGTTTAACTTCTATTTGCGTGGT-AGTT 65
ON8415B_repeat2 CTTTCTAAGTTCAAAAATCAAAAAATATCTTCTTTTGTGTTGTTGTTGTTGTTGTTAGT 65
ON8415B_repeat3 TTTCTAAGAAAAAGAAAAACAAAAATATCTTCTTTTGTGTTGTTGTTGTTGTTGTTAAC-AGCA 65

Fig. 5. Tandem repeats of 65 nucleotides are detected in the 5'-untranslated region (UTR) of *Grapevine leafroll-associated virus 3* (GLRaV-3) isolates. Shown here are **A**, a summary of the genomic location and copy numbers of these sequence repeats as well as sequence conservation between repeats in each variant group; **B**, an alignment of tandem repeats detected in ON8415A; and **C**, alignment of tandem repeats detected in ON8415B. Tandem repeats were identified by using the Tandem Repeats Finder Program (Benson 1999). TBD = to be determined.

positive-sense RNA viruses. Underlying mechanisms that govern the biogenesis and biological function of these tandem repeats in these viruses remain to be deciphered.

In addition to its paramount detrimental impact on viticulture and wine making, GLRaV-3 and other members of the genus *Ampelovirus* present significant interest as models of plant virology. Indeed, their genomes and 5'-UTRs are among the largest known of plant viruses, they contain exceedingly long internal noncoding regions (GLRaV-1 and GLRaV-3), they encode enigmatic AlkB domains proposed to repair methylated virus RNA (van den Born et al. 2008), they target mitochondria to form the replication complexes (Faoro 1997), and there are several other proteins and genetic features of unknown function that are unique to this group of viruses. Little is known concerning how members of the *Ampelovirus* genus interact with their grapevine host leading to severe diseases that threaten the grape and wine industries worldwide. By expanding the knowledge of genetic diversity of GLRaV-3 within a complex vine virome, this work contributes to inquiry into GLD mechanisms, as well as to its effective control in a young and rapidly growing grape and wine industry in Canada. In closing, the two novel and divergent GLRaV-3 isolates we report here add to the repertoire of genetic diversity, phylogenetics, as well as diagnosis of this economically very important virus. GLRaV-3 and other related members of the genus *Ampelovirus* are truly peculiar viruses that inspire curiosity, wonder, and investigation in a wide range of directions such as viral ecology, specificity in vector transmission, as well as pathological properties of different viruses and viral strains.

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