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Distinct Responses of *Vitis aestivalis* ‘Norton’ and *Vitis vinifera* ‘Kishmish Vatkana’ to Seven Viruses Revealed by Small RNA Sequencing

Susanne Howard, Sylvia Petersen, Adam Uhls, and Wenping Qiu[†]

Center for Grapevine Biotechnology, the W. H. Darr College of Agriculture, Missouri State University, Springfield, MO 65897

Accepted for publication 5 May 2021.

ABSTRACT

Grapevines are frequently infected by multiple viruses. Our previous study showed that Norton grapevine (*Vitis aestivalis*) is resistant to grapevine vein clearing virus (GVCV), a DNA virus in the family *Caulimoviridae*. To study the reaction of Norton to RNA viruses, we transferred seven RNA viruses to Norton from Kishmish Vatkana (KV) (*V. vinifera*) via graft transmission. We profiled viral small RNAs (vsRNAs) of the seven viruses and compared viral titers in Norton and KV. Total vsRNAs of grapevine leafroll-associated virus 1 (GLRaV-1), GLRaV-2, GLRaV-3, grapevine virus A (GVA), and grapevine Pinot gris virus (GPGV) were significantly less abundant in Norton than in KV, but total vsRNAs of grapevine fleck virus (GFKV) were more abundant in Norton than in KV. Total vsRNAs of grapevine rupestris stem pitting-associated virus (GRSPaV) were not different between Norton and KV. Grafting direction of Norton to KV or KV to Norton

did not affect the quantity of vsRNAs. The genome coverage of GLRaV-1, GLRaV-2, GLRaV-3, and GVA vsRNAs was lower in Norton than KV. The 21- and 22-nucleotide classes of vsRNAs were predominant for all seven viruses. Virus quantification by quantitative PCR indicated that GLRaV-1 was undetectable in Norton; GLRaV-2, GLRaV-3, and GVA were less abundant in Norton; and GFKV was more abundant in Norton than in KV. These results demonstrated that Norton grapevine suppresses GLRaV-1, GLRaV-2, GLRaV-3, and GVA but supports GFKV in comparison with KV. This study revealed new facets of complex molecular interactions between grapevines and multiple viruses.

Keywords: grapevine, microbiome, Norton, plant pathology, resistance, virology, virome, virus

Grapevines (*Vitis* spp.) host more than 86 viruses, which threaten grape production in all viticultural areas (Martelli 2018). A group of six viruses, referred to as grapevine leafroll-associated viruses (GLRaVs), are the most widely epidemic and damaging in vineyards worldwide. The grapevine leafroll disease is frequently associated with infection of more than one virus, which increases disease severity and complicates diagnosis (Naidu et al. 2015). Although much research focuses on diagnosis, genetic diversity, and epidemics of grapevine viruses, less is known about viral resistance. One factor

is that genetic resources of grapevine germplasm that are resistant against viruses are scarce (Martelli 2014; Oliver and Fuchs 2011). For instance, no *Vitis* accessions were found that show resistance to grapevine leafroll-associated virus 1 (GLRaV-1) and GLRaV-3 after assessment of 223 *Vitis* accessions (Lahogue and Boulard 1996). No *Vitis* plants resistant to other GLRaVs have been documented, either (Laimer et al. 2009; Maree et al. 2013; Naidu et al. 2015). Another factor is that conducting research on virus resistance in a woody perennial plant is challenging and time consuming due to experimental duration and field limitation.

In a grapevine importation project, *Vitis vinifera* ‘Kishmish Vatkana’ (KV) was found to be infected with multiple viruses. Specifically, enzyme-linked immunosorbent assay (ELISA) diagnostic assays indicated that this vine was infected with grapevine fanleaf virus (GFLV), grapevine fleck virus (GFKV), GLRaV-1, GLRaV-3, and grapevine virus A (GVA) (W. Qiu, unpublished results). These viruses belong to four families representing distinct genomic structures and compositions as well as different processes of replication and movement. A source of multiple viruses in a single grapevine provides an opportunity for assessing a grape cultivar’s resistance to multiple viruses and for studying potential mechanisms underlying

S. Howard and S. Petersen made equal contributions to this project.

[†]Corresponding author: W. Qiu; wenpingQiu@missouristate.edu

Funding: Continuous support of our research program is provided by the Missouri Grape and Wine Board.

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

The author(s) declare no conflict of interest.

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grapevine–virus interactions through a comprehensive profiling of viral small RNAs (vsRNAs).

vsRNAs (i.e., short RNAs) are hallmarks of gene silencing against virus infection in plants (B. Zhang et al. 2019). Gene silencing targets viral double-stranded RNAs to produce primary vsRNAs, which are then recruited by the RNA-induced silencing complex to slice viral single-stranded RNAs. Therefore, vsRNAs consist of small interfering RNAs (siRNAs) and residual RNAs that are degradation products of viral RNAs. RNA sequencing (RNAseq) of vsRNAs by high-throughput sequencing enables simultaneous detection of all potential viruses in a plant (Pooggin 2018). It also aids virome construction in a plant infected with a virus mix and reveals genetic signatures of RNA interference (RNAi) pathways and genetic diversity of viral quasispecies (Pooggin 2018). This type of analysis has been used to find viruses and assemble viral genomes in grapevines (Czotter et al. 2018), and is particularly suitable for high-throughput detection of multiple grapevine viruses (Czotter et al. 2018; Eichmeier et al. 2016, 2019; Massart et al. 2019). For instance, 3 GFLV genomes, 11 grapevine rupestris stem-pitting associated virus (GRSPaV) genomes, and 6 viroids were assembled de novo by high-throughput sequencing of small RNAs (Hily et al. 2018). Five viruses, including GLRaV-1, GLRaV-3, GVA, grapevine virus B (GVB) and GRSPaV, and three viroids were found in a single Riesling grapevine (Xiao et al. 2019), while three viruses (GLRaV-1, GVA, and GRSPaV) and two viroids were discovered in a Pinot Noir grapevine (Beuve et al. 2018).

Small RNAs move through the entire plant (Maizel et al. 2020; B. Zhang et al. 2019) and can trigger systemic silencing that regulates development and antiviral defense (Baulcombe 2004). In one study, it was shown that when a susceptible scion of sweet cherry was grafted onto a rootstock that generates short hairpin RNAs of prunus necrotic ringspot virus (PNRSV), the scion became resistant to PNRSV. This is a confirmed case of long-distance transport of siRNAs from rootstock to scion in a grafted woody plant (Zhao and Song 2014). Although it is likely that 22-nucleotide (nt) siRNAs are a mobile signal for systemic silencing in *Arabidopsis thaliana* and *Nicotiana benthamiana* plants (X. Zhang et al. 2019), identity of mobile RNAs for inducing systemic silencing and their trafficking routes and modes remains largely unclear in woody perennial plants.

Norton is a grapevine cultivar of *V. aestivalis* that is native to the North American continent. It is resistant to grapevine vein clearing virus (GVCV), a DNA virus in the family *Caulimoviridae* (Qiu et al. 2020). Because it is unknown whether Norton is resistant to RNA viruses, we asked two questions. (i) Is Norton resistant to any RNA virus? (ii) Can small RNA profiling reveal some features underlying Norton–virus interactions? Answering these basic questions will aid in understanding molecular mechanisms of grapevine–virus interactions.

MATERIALS AND METHODS

The table grape cultivar KV (*V. vinifera*) was introduced to Missouri State Fruit Experiment Station at Mountain Grove, MO from Hungary in 2006, as a powdery mildew-resistant grape cultivar in a research project. The originally imported cuttings were propagated and maintained in a quarantine greenhouse. In testing required for quarantine purposes, GFLV, GfKv, GVA, GLRaV-1, and GLRaV-3 were detected in the KV grapevine by the ELISA kit from BIOREBA AG (Christoph Merian-Ring 7, CH-4153 Reinach BL1, Switzerland). In this study, KV was used as a source of multiple viruses.

Norton has been grown in the United States for more than 180 years. Its genetic background derives mainly from wild *V. aestivalis* (approximately 75%) (Hammers et al. 2017). One Norton vine was produced by microshoot tissue culture for the National Clean Plant Network–Grapes project and maintained in a screened greenhouse.

The mother vine Norton-VFM131 was analyzed for GFLV, tomato ringspot virus, tobacco ringspot virus, arabis mosaic virus, GfKv, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-7, GLRaV-4 strains 5 and 9, GVA, GVB, and GRSPaV by reverse-transcription PCR (RT-PCR), and GVCV by PCR, and found to be free of these 16 viruses. Subsequent RNAseq analysis of Norton-VFM131 virome did not detect viruses except for GRSPaV, hop stunt viroid (HSVd), and grapevine yellow speckle viroid (GYSVd).

Transmission by grafting. Wedge grafting was used to inoculate Norton-VFM131 with multiple viruses. Green cuttings of KV with one node were grafted as scion onto two-node green cuttings of Norton-VFM131 as rootstock. In the reciprocal graft, Norton-VFM131 was used as scion and KV as rootstock. After dipping the bottom end of the rootstocks into Rhizopon AA #1 rooting powder (The Hortus USA Corp., Earth City, MO, U.S.A.), the grafted vines were kept under mist until rooted and then grown under greenhouse conditions. Graft unions were visually examined to ensure healing of vascular tissue between scion and rootstock. For each graft direction, three grafted grapevines were used as biological replicates for subsequent analysis.

RNA extraction, RNAseq, and data processing. Total RNA was extracted from 200 mg of leaf tissue sampled 5 months after grafting following a modified protocol (Fung et al. 2008; Malnoy et al. 2001). Leaf tissue was ground in liquid nitrogen and the powder was mixed with 1.5 ml of extraction buffer (Fung et al. 2008) and incubated at 65°C for 5 min; then, the leaf emulsion was placed at –80°C for 24 h. Upon thawing at 40°C for 10 min, the emulsion was centrifuged at 16,000 × *g* at 4°C for 30 min. The supernatant was removed and then centrifuged at 16,000 × *g* for 5 min. An equal volume of chloroform was added to the supernatant, vortexed vigorously, and incubated on ice for 5 min before centrifugation at 16,000 × *g* for 15 min. Total RNA was precipitated by 2.4 M LiCl at –20°C for 2 h, then collected by centrifuging for 30 min. The pellet was washed with 80% ethanol three times, dried, and dissolved in 30 µl of nuclease-free water. Total RNA was treated with DNase (DNA-free DNA Removal Kit Invitrogen, Carlsbad, CA, U.S.A.). RNA quantity and quality were assessed by gel electrophoresis and Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, U.S.A.). Total RNA samples that had an RNA integrity number of greater than seven were subjected to RNAseq procedures.

Small RNA libraries were made at Admera Health (South Plainfield, NJ, U.S.A.) using the NEBNext Multiplex Small RNA Library Prep Set for Illumina sequencing (New England Biolabs Inc., Ipswich, MA, U.S.A.). The library preparation and size selection followed the manufacturer's protocol. The library was sequenced on the Illumina HiSeq for 150 cycles with an insert size of 15 to 35 nt. After primer sequences were removed with Cutadapt 1.9.1, the reads were checked for quality using a cutoff Phred score of 20. Reads shorter than 18 nt in length were removed. This resulted in approximately 26 to 30 million reads, which were used in the following analyses (Supplementary Tables S1 and S2).

All reads were used with BLAST 2.6.0+ to find one match each in the complete NCBI database. The search was restricted to virus matches. This untargeted search resulted in matches to GfKv, GLRaV-1, GLRaV-2, GLRaV-3, GVA, grapevine Pinot gris virus (GPGV), GRSPaV, and two viroids, HSVd and GYSVd. No reads aligning to GFLV were found. The accessions with the largest number of matching reads were selected as reference sequences for further analyses (Supplementary Table S3).

Bowtie 2 was used to align all reads to the reference sequences with a maximum mismatch of two. The number of reads aligning to each virus or viroid plus the total number of virus-related reads is shown in Supplementary Table S1. Reads per million were calculated to normalize these numbers for sequencing depth (Supplementary Table S2).

IGV V1.7 was used to visualize the alignments of the reads and to export a consensus sequence for each sample and virus. From that consensus sequence, the percentage of reference genome coverage was calculated for each virus. This coverage contained contigs as well as single reads and was higher than coverage based only on contigs.

Analysis of the genome coverage by vsRNAs. Using the information from the read alignment files (SAM format), we constructed a coverage map for each virus. The coverage map allowed us to locate areas that were not covered by vsRNAs (gaps), calculate numbers and length of contigs and gap length, and compare these values between the two cultivars using a Student's *t* test (Supplementary Table S4). For this purpose, all aligned reads were used, including those that were not incorporated into contigs. Contigs are defined as areas of continuous coverage at least 23 nt long. In addition to these assembly descriptors, we located the highest peak (maximum coverage) and the gaps with the maximum length.

We constructed the coverage maps of the aligned reads to visually observe differences between the seven viral genomes and between the two cultivars (Supplementary Fig. S1). In addition, a map of the first derivative of the coverage, scaled to 100% of the highest peak, was constructed to compare each of the seven viral genomes between the two cultivars on the same scale (Supplementary Fig. S1). The first derivative was calculated as follows: [(coverage at position $n + 2$) – (coverage at position n)]/2.

RT and quantitative real-time PCR. Total RNA (2 µg) was reverse-transcribed to cDNA with random hexamer primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, U.S.A.). The quantity of virus-specific fragments was measured by quantitative real-time PCR (qPCR) using SYBR Green in the MX3005P system (Stratagene, San Diego, CA, U.S.A.). The 12.5-µl reaction contained 5 µl of 1:50 diluted cDNA as template, 6.25 µl of Brilliant II SYBR Green QPCR Master mix (Agilent Technologies), 30 nM reference dye (ROX), each primer at optimized concentration, and water to 12.5 µl. The optimal primer concentration for each virus was determined by using a primer matrix, and the optimal annealing temperature was found using VeriFlex PCR (Applied Biosystems). Primer concentrations and annealing temperatures are listed in Supplementary Table S3. Sequences of primers were adopted from previous studies for GLRaV-1 (reverse primer), GLRaV-3 and the 18S ribosomal RNA (rRNA) gene (Osman et al. 2007), GPGV (Morán et al. 2018), and the β -actin gene (Bruissson et al. 2017). Remaining primers were designed in this study based on the viral genomes assembled from vsRNAs. All samples and standard curves were run in triplicate. Thermal cycling consisted of 95°C for 10 min, 40 cycles of 95°C for 30 s, and the optimal annealing and extension temperature for each primer pair for 1 min. The dissociation curve was plotted from one cycle at 95°C for 30 s and a temperature gradient from 55 to 95°C. All dissociation curves of qPCR products showed a single peak. The qPCR-amplified DNA fragments were also subjected to Sanger sequencing to ascertain the identity of each viral fragment. PCR efficiency and the R^2 value from the standard curve were calculated using the absolute quantification method of MxPro software.

The standard curve for each virus and reference gene was created using DNA fragments synthesized by Eurofins (Eurofins Genomics, Louisville, KY, U.S.A.) or gel-purified PCR products. DNA concentration was converted to copy number based on the molecular weight and length of each fragment and the Avogadro constant (6.023×10^{23} molecules/mol). The standard curve for each virus consisted of one dilution point at 1×10^7 and fivefold serial dilutions from 1×10^6 to 12.8 copies. The standard curve for the β -actin gene consisted of eight points of 10-fold serial dilution from 1×10^8 to 10 copies, and for 18S rRNA from 5×10^8 to 50 copies. The abundance of

β -actin and the 18S rRNA gene was not significantly different across graft direction and cultivar. The number of β -actin genes was used as reference to calculate the relative number of viral fragments because of its relatively smaller numbers.

The copy numbers for each virus-specific fragment were retrieved from the MxPro software and calibrated by the amplification efficiency of each primer set. The resultant copy number was expressed as copy number per million β -actin copies.

Statistical analysis. All datasets were checked for normal distribution by the Kolgorow-Smirnov test of NCSS, version 07.1.21 (NCSS, LLC, Kaysville, UT, U.S.A.) following the analysis->descriptive statistics->normality tests procedure. For data sets that were normally distributed, the NCSS analysis of variance (ANOVA) general linear models procedure followed by Fisher's least significant difference test was used to determine significant differences between means with $\alpha = 0.05$. For data sets that were not distributed normally, the NCSS Wilcoxon rank sum test was used to determine significant differences with $\alpha = 0.05$, which is part of the *t* test two-sample procedure. The linear regression and correlation method of NCSS was used to calculate the Pearson correlation coefficient for correlation between copy number and RPM.

RESULTS AND DISCUSSION

vsRNAs were less abundant in Norton than in KV. In this study, we inoculated Norton grapevines, as scion or rootstock, with multiple viruses from a source KV grapevine via graft transmission. With these materials, RNAseq was performed for the analysis of 12 viromes (3 Norton scions, 3 Norton rootstocks, 3 KV scions, and 3 KV rootstocks). A range of 18- to 40-nt small RNAs was selected as the focal group; the number of total clean reads and viral reads is provided in Supplementary Table S1. As such, small RNAs were detected that aligned with the genomes of seven viruses (GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GVA, GPGV, and GRSPaV) and two viroids (HSVd and GYSVd) (Supplementary Table S1). These virus-derived small RNAs are collectively referred to as vsRNAs. The seven viruses belong to three families and six genera but all use single-stranded, plus-sense RNA as genome (Table 1).

We calculated the percentage of vsRNAs of each virus and viroid among the total vsRNAs and compared them between the two cultivars (Fig. 1). The vsRNAs of GLRaV-1 (35%), GPGV (27%), and GLRaV-3 (8%) were the three most abundant in KV (Fig. 1A). In contrast, the vsRNAs of GPGV (25%), HSVd (21%), GYSVd (17%), and GFkV (16%) were the most abundant in Norton (Fig. 1B). The percentage of GPGV vsRNA was similar in KV and in Norton. Virome composition clearly differed between KV and Norton. Other virome studies showed that, in Riesling grapevine, the three most abundant viruses were GLRaV-3 (84.9%), GVB (11.5%), and GVA (1.6%) (Xiao et al. 2019); in Pinot Noir grapevine, they were GLRaV-2 (43%), GLRaV-3 (38%), and GRSPaV (7%); in hybrid Vidal Blanc, they were GRSPaV (61%), GLRaV-2 (18%), and GLRaV-3 (13%); and in hybrid Marechal Foch, they were GLRaV-3 (43%), GRSPaV (37%), and grapevine virus E (13%) (Fall et al. 2020). These results support that GLRaV-1, GLRaV-2, and GLRaV-3 contribute a large portion to the viral populations in grapevines of *V. vinifera* and hybrid grape, and the genetic background of each grape cultivar influences the composition of vsRNA profiles. Because HSVd and GYSVd are universally distributed and well analyzed in grape cultivars (Czotter et al. 2018; Di Serio et al. 2017), we did not perform further analysis on the two viroids.

We analyzed the reads per million (RPM) of total vsRNAs derived from the seven viruses (Table 2). In total, 4,789 RPM vsRNA was obtained in Norton and 12,677 RPM vsRNA in KV. Total vsRNA was significantly less in Norton than in KV (Table 2). This

observation prompted us to compare abundance of vsRNAs for each virus between both cultivars. The RPM of GFkV vsRNA was significantly higher in Norton than in KV, indicating that more GFkV vsRNAs accumulated in Norton than KV (Table 2, top panel). In contrast, the RPM of GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GPGV vsRNA was significantly lower in Norton than in KV (Table 2, top panel). There was no difference in the RPM of GRSPaV vsRNAs between Norton and KV.

Grafting direction has no significant effect on total vsRNA numbers. We used one-way ANOVA to compare total vsRNAs between rootstock and scion in both cultivars. There was no significant difference in the total RPM between Norton rootstock and scion (Table 3) but RPM of vsRNAs was higher in KV scion than in rootstock (Table 3). This analysis indicated that vsRNAs of the seven viruses translocated similarly to Norton scion or rootstock, independently of the direction of virus inoculum (Table 3). Because Norton was free of GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GPGV prior to graft transmission (W. Qiu, unpublished result), it is reasonable to conclude that vsRNAs of these viruses in Norton were products of direct translocation to Norton from KV and de novo generation of whole viruses in Norton.

Genome coverage by vsRNAs differs in Norton and KV. We applied one-way ANOVA to analyze differences in the coverage of vsRNAs for each viral genome. Genome coverage of GLRaV-1, GLRaV-2, GLRaV-3, and GVA by vsRNAs was lower in Norton as scion or rootstock than in KV (Fig. 2). There was no difference in the coverage of vsRNAs for GFkV, GPGV, and GRSPaV between Norton and KV regardless of graft direction (Fig. 2).

In the grafting set of Norton as scion and KV as rootstock, the coverage of vsRNAs for GLRaV-1, GLRaV-3, and GVA genomes was significantly lower in Norton than in KV (Fig. 2A). vsRNAs covered 30.7% in Norton versus 97.3% in KV of GLRaV-1 genome; 66.5% in Norton versus 97.2% in KV of GLRaV-3 genome; 34.8% in Norton versus 60.6% in KV of GVA genome (Fig. 2A).

In the grafting set of KV as scion and Norton as rootstock, the coverage of vsRNAs for GLRaV-1, GLRaV-2, GLRaV-3 and GVA genomes was lower in Norton rootstock than in KV scion (Fig. 2B). In this case, vsRNAs covered 55.0% in Norton versus 97.6% in KV of GLRaV-1 genome; 82.0% in Norton versus 96.5% in KV of GLRaV-3 genome; 39.2% in Norton versus 61.3% in KV of GVA genome.

The significant difference in RPM of GFkV vsRNAs between Norton and KV disappears when comparing genome coverage (Table 2; Fig. 2). Although Norton rootstock had two times higher RPM than KV scion (Table 2), the genome coverage differed by

only approximately 3%. This was due to many reads covering the same region of the reference sequence.

Comparison of Norton scion and rootstock showed that coverage of GLRaV-1 (30.7%) genome by vsRNAs was lower in Norton scion than in Norton rootstock (55%). Similarly, coverage of GLRaV-3 by vsRNAs (66.5%) was lower in Norton scion than in rootstock (82%) (Fig. 2). Biological implications of differences in the coverage in scion versus rootstock merits further investigation.

The above analyses clearly indicated that the vsRNAs of GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GPGV were significantly less abundant while GFkV vsRNAs were more abundant in Norton than in KV (Table 2). Genome coverage by vsRNAs for each virus differed between the cultivars but no full-length genome could be reconstructed by vsRNAs (Fig. 2). For instance, the genome coverage of GLRaV-1 and GLRaV-3 by vsRNAs was 97.3 and 97.2%, respectively, in KV rootstock (Fig. 2A). The genome coverage of GLRaV-2 was less than 30% in KV rootstock (Fig. 2A). It was reported that using rRNA-depleted total RNA or enriched double-stranded RNA in RNAseq is more effective in assembling whole genomes of plant viruses than using small RNAs (MacIot et al. 2020; Xiao et al. 2019). We used small RNAs to construct cDNA libraries and perform RNAseq in this study. Uneven distribution of vsRNAs along the whole genome of each virus may contribute to the incomplete genome assembly of the seven viruses, in agreement with a recent report (Sidharthan et al. 2020). Because it is not possible to reconstruct the full-genome of grapevine viruses by using vsRNAs, additional evidence is required to verify whether the viruses with low abundance and incomplete genome coverage of vsRNAs are replicated in Norton.

RT-qPCR confirmed that Norton suppresses GLRaV-1, GLRaV-2, GLRaV-3, and GVA but supports GFkV and GPGV. To verify the RNAseq results, we performed qPCR to assess viral titers of GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GPGV. The numbers of subgenomic RNAs (sgRNAs) generated by each virus are different (Table 1) and, thus, influence the copy number of virus-specific fragment amplified by qPCR. In the qPCR assay, we used one set of primers that was designed from one specific region of each viral genome (Supplementary Table S3). We made cDNA for each virus using random primers, and cDNA was produced from both genomic and sgRNAs. Because the numbers of sgRNAs to which each primer set is aligned are different (Supplementary Table S3), the quantity of DNA fragments measured by qPCR is a sum of genomic and sgRNA-derived cDNA molecules for GLRaV-1, GLRaV-2, GLRaV-3, GPGV, and GVA. The exception is GFkV, for which two primers align only to the genome. Because abundance of sgRNAs varies among viruses, viral titers of each virus determined

TABLE 1
Classification and genome features of the seven viruses analyzed in this study^z

Viruses	Family	Genus	Genome	Genome size (nt)	Number of sgRNAs
<i>Grapevine fleck virus</i> (GFkV)	<i>Tymoviridae</i>	<i>Marculavirus</i>	ss (+) RNA	Approximately 7,500	2
<i>Grapevine leaf roll-associated virus 1</i> (GLRaV-1)	<i>Closteroviridae</i>	<i>Ampelovirus</i>	ss (+) RNA	12,394–18,659	8
<i>Grapevine leaf roll-associated virus 2</i> (GLRaV-2)	<i>Closteroviridae</i>	<i>Closterovirus</i>	ss (+) RNA	Approximately 16,500	7
<i>Grapevine leaf roll-associated virus 3</i> (GLRaV-3)	<i>Closteroviridae</i>	<i>Ampelovirus</i>	ss (+) RNA	13,400–18,600	9
<i>Grapevine virus A</i> (GVA)	<i>Betaflexiviridae</i>	<i>Vitivirus</i>	ss (+) RNA	7,400–7,600	3
<i>Grapevine Pinot gris virus</i> (GPGV)	<i>Betaflexiviridae</i>	<i>Tricovirus</i>	ss (+) RNA	7,259	2
<i>Grapevine rupestris stem pitting-associated virus</i> (GRSPaV)	<i>Betaflexiviridae</i>	<i>Foreavirus</i>	ss (+) RNA	8,725	3

^z Abbreviations: nt = nucleotide, sgRNA = subgenomic RNA, and ss (+) RNA = single-stranded, plus-sense RNA.

Fig. 1. Distribution of viral small RNAs (vsRNAs) of each virus among the total vsRNAs that were aligned to all seven viruses and two viroids. Number of total vsRNAs in each cultivar was calculated by summing all vsRNAs of the six samples independent of grafting direction. **A**, Percentage of each viral vsRNAs in grape cultivar Kishmish Vatkana. **B**, Percentage of each viral vsRNAs in grape cultivar Norton. GFkV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A; GPGV = grapevine Pinot gris virus; GRSPaV = grapevine rupestris stem pitting-associated virus; HSVd = hop stunt viroid; and GYSVd = grapevine yellow speckle viroid.

by qPCR are not comparable in the present study. The Pearson correlation coefficient analysis between vsRNA RPM and copy number by qPCR found that there is a good correlation between the two numbers for GLRaV-1 ($r = 0.984$), GLRaV-2 ($r = 0.793$), GLRaV-3 ($r = 0.924$), and GFkV ($r = 0.813$) but not for GVA ($r = 0.558$) and GPGV ($r = 0.125$).

During viral transcription and gene expression, GFkV produces two sgRNAs (Sabanadzovic et al. 2017). The two primers in the qPCR assay for GFkV are in the coat protein (CP) region (Supplementary Table S3), allowing quantification of only GFkV genomic RNA molecules. The genome number of GFkV was over three times more abundant in Norton than in KV (Table 4), which was consistent with the abundance of vsRNAs (Table 2).

GLRaV-1 generates eight sgRNAs in its infectious cycle (Donda et al. 2017; Naidu 2017). The two primers used in qPCR were located in the HSP70 open reading frame (ORF) on the GLRaV-1 genome and, therefore, allowed detection of genomic RNA and three sgRNAs (Supplementary Table S3). GLRaV-1 was not detected in Norton scion or rootstock whereas the copy number of GLRaV-1-specific fragments per million β -actin was 2,449 in KV (Table 4). Conventional RT-PCR using another set of primers located in the CP gene did not detect GLRaV-1 in Norton, either (data not shown). These results demonstrated that Norton suppresses graft-transmissible GLRaV-1. GLRaV-1 had relatively lower titer in KV in comparison with GLRaV-3 (Table 4); this may be one factor explaining why GLRaV-1 was not detected in Norton.

GLRaV-2 produces seven sgRNAs (Angelini et al. 2017). The two primers for GLRaV-2 align to the p19 ORF and, thus, allow quantification of genomic RNA and seven sgRNAs (Supplementary Table S3). The copy number of GLRaV-2-specific DNA fragment per million β -actin was substantially higher in KV than in Norton (Table 4).

GLRaV-3 produces nine sgRNAs (Burger et al. 2017; Maree et al. 2013). The primers used for GLRaV-3 were located in the HSP70 ORF that allows quantification of genomic and three sgRNAs (Supplementary Table S3). The copy number of GLRaV-3-specific

fragments per million β -actin in Norton was 77, which was significantly less than the 13,331 copies in KV (Table 4). This result was consistent with the vsRNA reads (Table 2) and the conventional RT-PCR result using a set of primers in the CP gene (data not shown).

GVA generates three sgRNAs (Minafra et al. 2017). The primer set for GVA aligned to nucleotides 7,055 and 7,202 and, thus, allowed detection of genomic and three sgRNAs (Supplementary Table S3). The copy number of GVA-specific fragments per million β -actin was 1,790 in Norton and 707,195 in KV (Table 4).

GPGV produces two sgRNAs (Saldarelli et al. 2017). The primer set for GPGV was derived from nucleotides 6,516 to 6,744 and, thus, allowed detection of genomic and two sgRNAs. The copy number of GPGV-specific fragments was not significantly different between Norton and KV (Table 4).

Because the genomes of GRSPaV were almost fully assembled from vsRNAs, and coverage was not significantly different between the two cultivars, we did not perform RT-qPCR assay on GRSPaV.

GLRaV-1 was not detected in Norton by RT-qPCR, suggesting that, indeed, no sgRNAs were produced in Norton; thus, it is reasonable to conclude that Norton highly suppressed GLRaV-1. In the case of GLRaV-2 and GLRaV-3, copy numbers of virus-specific DNA fragments were significantly less in Norton than in KV, suggesting that Norton substantially suppresses GLRaV-2 and GLRaV-3 (Table 4). GLRaV-1 and GLRaV-3 belong to the genus *Ampelovirus* (Burger et al. 2017; Naidu 2017) whereas GLRaV-2 is a member of the genus *Closterovirus* (Angelini et al. 2017). Their genomic structures and organizations are different; however, the results support the idea that Norton suppresses all three viruses. Much progress has been achieved on the genetic, genomic, and epidemiological understanding of GLRaVs (Naidu et al. 2015). Little is known about how grapevines defend against viruses in the *Ampelovirus* and *Closterovirus* genera that consist of diverse species and variants (Angelini et al. 2017; Burger et al. 2017; Naidu 2017). The present study did not discern whether lower virus population is due to blocking virus movement or suppression of viral replication. No detection of GLRaV-1 in

TABLE 2
Statistical analysis of small viral RNA reads per million by cultivar and by graft position^z

Cultivar, position	GFkV	GLRaV-1	GLRaV-2	GLRaV-3	GVA	GPGV	GRSPaV	Total
Cultivar								
Kishmish Vatkana	561.17 ± 208 b	5,233.5 ± 3,437 a	782 ± 520 a	1,082 ± 636 a	421.5 ± 148 a	3,820 ± 1,919 a	778.33 ± 459 a	12,677 ± 4,408 a
Norton	1,199.2 ± 299 a	89.833 ± 114 b	17 ± 20 b	264.2 ± 141 b	104.5 ± 161 b	1,943 ± 731 b	1,171.3 ± 565 a	4,789 ± 717 b
Position								
Scion	878 ± 285 a	3,521 ± 4,665 a	318 ± 404 a	463.3 ± 312 a	315.2 ± 238 a	3,455 ± 2,093 a	1,104 ± 596 a	10,054 ± 6,064 a
Rootstock	882.33 ± 544 a	1,802.3 ± 2,043 a	481 ± 789 a	882.3 ± 789 a	210.8 ± 212 a	2,308 ± 1,076 a	845.67 ± 478 a	7,413 ± 4,039 a

^z GFkV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A; GPGV = grapevine Pinot gris virus; and GRSPaV = grapevine rupestris stem pitting-associated virus. Different letters indicate significant difference at $\alpha = 0.05$.

TABLE 3
Statistical analysis of small viral RNA reads per million by combination of cultivar and graft position^y

Combination ^z	GFkV	GLRaV-1	GLRaV-2	GLRaV-3	GVA	GPGV	GRSPaV	Total
KV-R	413.3 ± 152 c	3,465.3 ± 1,455 ab	934.7 ± 697 a	1,421 ± 814 a	367 ± 194 ab	2,736.7 ± 1,305 ab	693 ± 274 a	10,031 ± 4,357 b
KV-S	709 ± 139 bc	7001.7 ± 4248 a	628.7 ± 344 ab	742 ± 63 ab	476 ± 90 a	4,902.3 ± 1,995 a	863.7 ± 656 a	15,322 ± 2,930 a
Norton-S	1,047 ± 312 ab	40.3 ± 58 b	6.7 ± 4 b	184.7 ± 77 b	154.3 ± 236 c	2007 ± 826 b	1,344.3 ± 532 a	4,784 ± 233 c
Norton-R	1,351.3 ± 239 a	139.3 ± 147 b	27.3 ± 25 b	343.7 ± 157 b	55 ± 40 c	1,878.3 ± 802 b	998.3 ± 653 a	4,793 ± 1110 c

^y GFkV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A; GPGV = grapevine Pinot gris virus; and GRSPaV = grapevine rupestris stem pitting-associated virus. Different letters indicate significant difference at $\alpha = 0.05$.

^z KV = Kishmish Vatkana, R = rootstock, and S = scion.

Norton may also be a result of low virus titer in the source vine of KV or the mere 5-month duration of graft transmission. However, these findings open a new avenue and stimulate inquiry into grapevine's defense mechanisms against viruses that has been not fully explored. No germplasm of *Vitis* spp. showing resistance or tolerance to

GLRaV-1 and GLRaV-3 was identified (Laimer et al. 2009; Oliver and Fuchs 2011), and *V. aestivalis* was not included in a 1996 study of virus resistance in *Vitis* spp. (Lahogue and Boulard 1996). Because grapevine leafroll disease causes substantial losses to grape and wine production worldwide (Naidu et al. 2014), finding a virus-resistant

Fig. 2. Comparison of percentages of each viral genome assembled from viral small RNAs (vsRNAs) in scion and rootstock of grape cultivars Kishmish Vatkana and Norton. **A**, Norton was grafted as scion (Norton-S) onto Kishmish Vatkana rootstock (KV-R) and **B**, Kishmish Vatkana was grafted as scion (KV-S) onto Norton rootstock (Norton-R). GFkV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A; GPGV = grapevine Pinot gris virus; and GRSPaV = grapevine rupestris stem pitting-associated virus. Bar denotes standard errors of means of three biological replicates; statistically significant differences are indicated by different letters, determined by analysis of variance ($P < 0.05$).

grapevine provides an opportunity for potential breeding of virus-resistant cultivars.

Genome assembly features of each virus differ between Norton and KV. In addition to the general comparisons in Figure 2, we performed detailed analysis of genome assembly features for each virus and compared these features between the two cultivars in Supplementary Figure S1 and Supplementary Table S4. Detailed descriptions of these features are also provided in Supplementary Document S1. Here, we report a salient summary. We found that, even with high coverage, gaps remained in the assembled genomes. All of the gaps were located in the replicase ORFs, except in GLRaV-3, where it was found in ORF 12 and the untranslated regions. Hotspots (regions with very high coverage) were also located in the replicase ORFs in GFkV and GPGV for both cultivars. For GLRaV-3, the hotspot was located in the replicase ORF only in KV and in ORF 11 in Norton. In GLRaV-1, GLRaV-2, and GVA, hotspots were located in the CP ORF and extended into neighboring ORFs. Overall, the distributions of gaps, hotspots, and regions with long contigs did not differ significantly between KV and Norton, even if only a few reads were present. This is possibly due to a constant flow of vsRNAs from KV to Norton.

We noticed three major features that merit further discussion. First, the maximum coverage of vsRNAs at specific nucleotide positions was found across the genome of each virus, although not evenly distributed. This could be biologically relevant, implying that a group of vsRNAs were preferentially amplified in the production of primary and secondary small RNAs. It might also be due to a technical factor that skewed amplification of a particular group of small RNAs during the construction of cDNA libraries. The second feature is the longest contig of each viral genome, suggesting that this region may form secondary structures that are conducive to RNAi and, thus, abundant vsRNAs were produced. Third, there were more frequent and longer gaps in the reference genome coverage of each virus in Norton than in KV. Significantly less abundance of vsRNAs in Norton contributed to the gap formation. Furthermore, gaps could also be a result of non-RNAi degradation of vsRNAs and genomic RNAs in host cells (Golyaev et al. 2019; Li and Wang 2019). It should also be noted that the seven viruses all encode potential viral suppressors. Mutual interactions of RNA silencing and suppression pathways for each virus can influence the overall dynamics of antiviral and counter-defense interactions.

Analysis of 21-, 22-, and 24-nt vsRNAs in Norton and KV. The distribution of 18- to 33-nt vsRNAs for each virus in Norton scion and rootstock as well as in KV scion and rootstock was compiled (Supplementary Table S5). We selected three vsRNA classes of 21-, 22-, and 24-nt vsRNAs to conduct a focal analysis because they are the most well characterized siRNAs in plants (Pooggin 2018). Consistent with previous discoveries on the classes of vsRNAs in grapevines (Alabi et al. 2012; Pantaleo et al. 2010), the most abundant was

the 21-nt class, followed by the 22-nt class for the seven viruses in the two cultivars (Table 5; Supplementary Fig. S2). These results suggested that grapevine DCL4 and DCL2 homologs play major roles in the biogenesis of vsRNAs as in other plants (Bouché et al. 2006; Donaire et al. 2009; Xie et al. 2004), and that all seven viruses, although in different genera and families (Table 1), are targeted in the same mode of action by the RNA silencing in both cultivars.

The percentage of the 21-nt class among vsRNAs of all seven viruses was higher in rootstock than in scion, whereas the 22-nt class was lower in rootstock than in scion (Table 5). This uneven distribution indicates underlying targeting by distinct DCL homologs and dynamics of vsRNA translocation. Distributions of 21-, 22-, and 24-nt classes differed for each virus (Fig. 3). For instance, the percentage of 22-nt class was approximately 26% for GFkV, which is consistent with a previous report (Pantaleo et al. 2010), and 23% for GPGV but only 7% for GLRaV-3. This reflects differential processing of viral RNAs in the RNA-silencing pathways as a result of distinct genome structures for each virus.

Detailed analysis of the three classes of vsRNAs between Norton scion and KV rootstock as well as KV scion and Norton rootstock is presented in Supplementary Figure S2. The most noticeable findings were that the percentage of 21-nt vsRNAs of GLRaV-1 was higher in Norton scion than in KV rootstock; percentage of 22-nt vsRNA was higher in KV scion than in Norton rootstock (Supplementary Fig. S2B). The significance of slightly different distribution of 21- and 22-nt classes of GLRaV-1 in scion and rootstock merits further investigation.

Conclusions. In this study, we provided evidence for several conclusions. (i) The total number of vsRNAs of the seven grapevine viruses was significantly less in Norton than in KV. Specifically, the total reads of GLRaV-1, GLRaV-2, GLRaV-3, GVA, and GPGV vsRNAs were substantially reduced in Norton but there were twice as many reads of GFkV vsRNAs in Norton than in KV.

TABLE 5
Statistical analysis of the percentages of 21-, 22-, and 24-nucleotide (nt) classes by cultivar and graft position^z

Cultivar, position	21 nt	22 nt	24 nt
Cultivar			
Kishmish Vatkana	62.63 a	15.75 a	0.77 a
Norton	60.89 a	13.80 b	0.63 a
Position			
Scion	59.53 b	15.90 a	0.77 a
Rootstock	63.99 a	13.57 b	0.64 a

^z Different letters indicate significant difference at $\alpha = 0.05$.

TABLE 4
Analysis of the copy number of viral fragments per million of β -actin by quantitative real-time PCR by cultivar and by graft position^z

Cultivar, position	GFkV	GLRaV-1	GLRaV-2	GLRaV-3	GVA	GPGV
Cultivar						
Kishmish Vatkana	50,758 $\pm$ 33,817 b	2,449 $\pm$ 2,212	1,187,957 $\pm$ 507,604 b	13,331 $\pm$ 14,292 b	707,195 $\pm$ 1,030,855 a	277,965 $\pm$ 124,451 a
Norton	196,777 $\pm$ 103,797 a	NA	56 $\pm$ 56 a	77 $\pm$ 82 a	1,790 $\pm$ 2,381 a	338,498 $\pm$ 219,290 a
Position						
Scion	131,791 $\pm$ 119,401 a	NA	537,399 $\pm$ 638,539 a	4,334 $\pm$ 4,609 a	600,267 $\pm$ 1,096,151 a	256,767 $\pm$ 17,7405 a
Rootstock	115,744 $\pm$ 101,486 a	NA	650,618 $\pm$ 856,833 a	9,073 $\pm$ 17864 a	108,718 $\pm$ 121,269 a	297,554 $\pm$ 200,338 a

^z GFkV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A, and GPGV = grapevine Pinot gris virus. Different letters indicate significant difference at $\alpha = 0.05$. NA = not analyzed due to no cycle quantification number for Norton.

Percentages of 21-, 22-, and 24-nt classes

Fig. 3. Statistical analysis of the percentages of 21-, 22-, and 24-nucleotide (nt) viral small RNA (vsRNA) classes between the seven viruses. GFKV = grapevine fleck virus; GLRaV-1, GLRaV-2, and GLRaV-3 = grapevine leaf roll-associated virus 1, -2, and -3, respectively; GVA = grapevine virus A; GPGV = grapevine Pinot gris virus; and GRSPaV = grapevine rupestris stem pitting-associated virus. Bar denotes standard errors of means of three biological replicates; different letters indicate significant differences at $\alpha = 0.05$.

(ii) The genome coverage by vsRNAs of GLRaV-1, GLRaV-2, GLRaV-3, and GVA was distinct between Norton and KV. (iii) GLRaV-1 was not detectable in Norton by RT-qPCR. The presence of vsRNAs and 42.9% genome coverage in Norton suggest that these vsRNAs were transported from KV. (iv) GLRaV-3 was much less abundant in Norton than in KV. (v) GFKV was more abundant in Norton than in KV. (vi) No genome of the seven viruses could be fully assembled, even combining vsRNAs from all 12 samples. Regions without coverage of even single reads often started at the same nucleotide position in all 12 samples for one virus (vii) When very few vsRNA were found in a sample, they were distributed along the whole viral genome. (viii) Classes of 21 and 22 nt were the most abundant vsRNA for all seven viruses regardless of their taxonomic classification. Therefore, our virome analyses in two grapevine cultivars of distinct genetic background reveals a complex picture of molecular grapevine–virus interactions. In conclusion, Norton resists GLRaVs to various degrees but supports GFKV accumulation to a high degree.

The Sequence Read Archive accession number for all RNA sequences obtained in this study is PRJNA666422.

ACKNOWLEDGMENTS

We thank H. Scholthof at Texas A&M University for critical reviewing of the manuscript and M. Rwahni and the team at Foundation Plant Services, University of California–Davis for providing services for detecting grapevine viruses to verify the results in this study.

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