The Relative Occurrence of Grapevine leafroll-associated virus 3 and Grapevine red blotch virus in Washington State Vineyards

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Abstract

Vineyard surveys were conducted for three consecutive seasons in eastern Washington State, the major grapevine-growing region in the state, to document the occurrence of *Grapevine leafroll-associated virus 3* (GLRaV-3) and *Grapevine red blotch virus* (GRBV). The majority of samples were collected from red-berried wine grape (*Vitis vinifera*) cultivars exhibiting symptoms of or suspected for grapevine leafroll (GLD) and red blotch (GRBD) diseases. A limited number of samples from white-berried cultivars were collected randomly due to the lack of visual symptoms. Samples were collected from a total of 2,063 grapevines from 18 red-berried cultivars and seven white-berried cultivars planted in eight American Viticultural Areas and tested for GLRaV-3 and GRBV using RT-PCR and PCR, respectively. The results showed 67.77% and 6.01% of total samples positive for GLRaV-3 and GRBV, respectively,

and 9.06% of samples positive for both viruses. About 17% of samples tested negative for the two viruses, but some of these samples were positive for GLRaV-2 and GLRaV-4. Overall results indicated that GLRaV-3 was more common than GRBV, independent of cultivars and the geographic origin of samples. Due to variability in symptoms in red-berried cultivars, virus-specific diagnostic assays were deemed necessary for reliable identification of GLRaV-3 and GRBV and to differentiate GLD and GRBD symptoms from those induced by biotic and abiotic stresses in vineyards. A multiplex PCR protocol was developed for simultaneous detection of GLRaV-3 and GRBV in grapevine samples. A global phylogenetic analysis of GRBV genome sequences revealed segregation of virus isolates from Washington State vineyards into two distinct clades, with the majority of isolates belonging to clade II.

The grape and wine industry represents the fourth largest agricultural sector in Washington State and contributed an estimated \$4.8 billion to the state's economy in 2013 (CAI 2015). Nearly 70 different wine grape cultivars (*Vitis vinifera*), at a ratio of 64% red- and 36% white-berried cultivars, are grown statewide in 14 American Viticultural Areas (AVAs, Washington State Wine 2018). With all but one of these AVAs located on the eastern side of the Cascade Mountains, nearly 98% of wine grape acreage is thus located in the arid cool climate of eastern Washington, which is considered highly suitable for producing excellent quality wine grapes.

Virus diseases have been recognized as one of the most significant constraints to sustainability of wine grapes in Washington State (Moyer

and O'Neal 2014). Due to their economic impact on the quality and production attributes of wine grapes in the state (Alabi et al. 2016; Naidu and Walsh 2015; Ricketts et al. 2017), knowledge about viruses is recognized as the foundation for improving the sanitary status of vineyards and deploying effective strategies to prevent the spread of viruses via planting materials and by vectors. During the past decade, several viruses and their strains have been documented in Washington State vineyards planted with wine grape (*V. vinifera*) and juice grape (*V. labruscana* 'Concord' and *V. labrusca* 'Niagara') cultivars (Alabi et al. 2010, 2011, 2014; Bahder et al. 2013a; Jarugula et al. 2010a, b; Martin et al. 2005; Mekuria et al. 2009; Poojari et al. 2013; Soule et al. 2006). Among them, *Grapevine leafroll-associated virus 3* (GLRaV-3; family *Closteroviridae*, genus *Ampelovirus*; Maree et al. 2013) was reported to be the most frequently encountered and wide spread across Washington State vineyards (Naidu 2011).

While grape growers in Washington State were grappling with GLRaV-3, the discovery of *Grapevine red blotch virus* (GRBV; family *Geminiviridae*, genus *Grablovirus*; Varsani et al. 2017) in vines showing red blotch symptoms (GRBD, Poojari et al. 2013) posed additional challenges to the management of virus diseases in vineyards. GRBV has been reported from several states in the U.S.A. (Al Rwahnih et al. 2013; Krenz et al. 2012; Thompson et al. 2018) and from Korea, Switzerland, Canada, and India (Krenz et al. 2014; Lim et al. 2016; Poojari et al. 2017), suggesting its presence in many grapevine-growing regions. However, none of these reports have considered the relative occurrence and significance of GRBD and GLD to vineyard health and production. Since GRBV and GLRaV-3 were reported to significantly affect fruit yield and quality attributes in self-rooted wine grape cultivars (Alabi et al. 2016; Poojari et al. 2013), knowledge of the distribution of these two viruses, with distinct genomes and replication strategies, would help to coordinate statewide implementation of decision-making strategies for controlling virus diseases in a holistic manner in Washington State vineyards.

Surveys are conducted to determine the economic significance of plant virus diseases and understand their epidemiological aspects to implement effective disease management strategies. Such surveys are especially critical in perennial crops, since these crops stay in the ground for many years and infected plants can serve as a constant source of inoculum for viruses to be spread in time and space by competent vectors present within and around the cropping system.

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Although the prevalence of GLRaV-3 was documented in several grapevine-growing regions in the United States and Canada (Fuchs et al. 2009; Krenz et al. 2014; Lim et al. 2016; Martin et al. 2005; Poojari et al. 2017; Sharma et al. 2015), few studies were conducted to simultaneously examine the occurrence of both GLRaV-3 and GRBV in vineyards largely because GRBV was reported only recently compared with GLRaV-3. In this study, therefore, a targeted survey was carried out for three consecutive seasons to determine the relative occurrence of GLRaV-3 and GRBV in Washington State vineyards with the aims of implementing sustainable strategies to mitigate negative impacts of GLD and GRBD.

Materials and Methods

Sample collection and extraction. Samples were collected from vineyards in eight AVAs in eastern Washington State. They are Yakima Valley, Horse Heaven Hills, Walla Walla Valley, Rattlesnake Hills, Columbia Valley, Lake Chelan, Wahluke Slope, and Red Mountain (Fig. 1). Vineyards were surveyed between late July and October during 2014, 2015, and 2016 seasons. Under Washington State conditions, this period corresponds to the post-*véraison* phase of berry development and maturity and represents the optimal period for monitoring symptoms in vineyards. Vineyards planted with red-berried wine grape cultivars were specifically targeted for this study, since these cultivars display the most obvious red leaf symptoms and are relatively easy to locate symptomatic vines in vineyards by visual observations. In addition, a limited number of samples from a few vineyards planted with white-berried cultivars were included in this study. Since these cultivars do not express symptoms similar to red-berried cultivars, leaf samples were collected from vines selected at random.

Samples were collected from vines in individual vineyard blocks essentially using the protocols described by Martin et al. (2005) and Jones et al. (2015). During the season, mature leaves were collected randomly from basal portions of canes from different parts of individual grapevines to account for the possible uneven distribution of the virus within a vine. Eight to 10 leaf samples collected from individual vines were pooled, placed into Ziploc bags, and stored in cool insulated containers until transported to the laboratory. During the dormant season, four cane samples were collected from individual vines of red-berried cultivars that were suspected to be virus infected during the preceding season based on the presence of red leaf symptoms. Information on the specific location of vineyards and names of growers were withheld for confidentiality. Approximately 250 mg of petiole sections from leaf samples and cambial scrapings from dormant canes were triturated in extraction bags using a semi-automated homogenizer (HOMEX 6, BIOREBA AG, Switzerland) as described earlier (Alabi et al. 2008; Jones et al. 2015). The extracts were either used immediately for virus detection or stored in small aliquots in a -80°C freezer for testing at a later time.

Detection of GRBV and GLRaV-3. Eight microliters of extract from individual samples were denatured (Alabi et al. 2008; Jones et al. 2015) and used as a template for detecting GRBV and

GLRaV-3 by PCR and reverse transcription (RT)-PCR, respectively. PCR assays were carried out using primers (Repfor: 5'-CAAGTCGTTGTAGATTGAGGACGTATTGG-3' and Reprev: 5'-AGCCACACCTACACGCCTTGCTCATC-3') to amplify a ~285 base pair (bp) fragment specific to a portion of the Rep gene of GRBV (Krenz et al. 2014). Single tube one step RT-PCR was performed using primers (MP7: 5'-GGGGDGGRACTTTCGAYGTSTC-3' and MP8: 5'-ATTGGACTRCCYTTYGGGAAAAT-3') to amplify a ~540 bp fragment specific to a portion of the heat shock protein 70-homolog (HSP70h) encoded by GLRaV-3 (Donda 2016). A subset of samples was tested for the presence of GLRaV-1, -2, and -4 using primers described in Osman et al. (2007). Grapevine samples previously tested positive for GLRaV-1, 2, -3, -4, and GRBV and negative for these viruses were included as positive and negative controls, respectively, for validation of test results. RT-PCR and PCR assays were carried out in 25 μl of total reaction volume using the Proflex PCR system (Thermo Scientific, Grand Island, NY). PCR products were resolved in a QIAxcel advanced system using QIAxcel DNA fast analysis kit and the data analyzed using QIAxcel screen gel software (Qiagen, Valencia, CA). For confirmation, an aliquot of PCR products from a subset of samples was resolved in 1.2% agarose gels pre-stained with GelRed (Biotium, UK) and visualized under UV light using a transilluminator (Bio-Rad Universal Hood, Bio-Rad Laboratories). The amplicons specific to GLRaV-3 and GRBV were subsequently cloned and sequenced, and nucleotide sequence of virus-specific cDNA clones determined as described earlier (Donda et al. 2017).

Multiplex RT-PCR for detection of GLRaV-3 and GRBV.

Multiplex PCR detection of GLRaV-3 and GRBV in individual grapevine samples was carried out using virus-specific primers (MP7 and MP8 for GLRaV-3 and Repfor and Reprev for GRBV) described above (Donda 2016; Krenz et al. 2014). Primers (nad5-s: 5'-GATGCTTCTTGGGGCTTCTTGTT-3' and nad5-as: 5'-CTCCA GTCACCAACATTGGCATAA-3') described earlier (Menzel et al. 2002) were included in each assay for amplifying a 181 bp DNA fragment specific to the NADH dehydrogenase subunit 5 (nad5). Multiplex PCR assays were performed in single tube one step format in 25 μl of total reaction volume containing 1 $\times$ PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl, pH 8.3) (Roche, Mannheim, Germany) with an additional 1.5 mM MgCl_2 (Roche), 1 $\times$ sucrose cresol red, 5 mM DTT (Life Technologies, Carlsbad, U.S.A.), 0.4 mM dNTPs (Roche), 4U RNase OUT (Life Technologies), 10 U Superscript III reverse transcription (Life Technologies), and 2 U Taq polymerase (Roche). The concentration of primers used in the multiplex PCR was 0.6, 0.1, and 0.2 μM for GLRaV-3, GRBV, and nad5 primers, respectively. Reverse-transcription was carried out at 52°C for 50 min followed by denaturation at 95°C for 5 min. This was followed by 35 sequential cycles of PCR, with each cycle of 95°C for 30 s, 56°C for 45 s, 72°C for 1 min, and a final extension performed at 72°C for 7 min. Positive and negative controls were included for validation of test results as described above. PCR assays were carried out using the Proflex PCR system and the amplified products resolved by agarose gel electrophoresis and visualized under UV light as described above.

Statistical analysis of the data. A generalized linear model (PROC GLM in SAS 9.3, SAS Institute, Inc. NC, U.S.A.) was used to perform the statistical analyses of the data. The proportions of vines that tested positive for GLRaV-3 and GRBV and for both viruses were calculated for each vineyard block by dividing the number of samples positive for each virus with the total number of samples collected from the vineyard block. Each vineyard block within individual AVAs was treated as a replication. Based on the year of planting, the vineyard blocks were classified into three age groups; namely, pre-2000s, 2001–2010, and 2011–2015, respectively. The proportion of vines in which GLRaV-3 and GRBV were detected, and coinfection of both viruses within and between AVAs, was calculated. The effect of age group on the presence of GLRaV-3 and GRBV in three age groups, either as single or coinfections, was also examined where the age of each group was considered as a fixed factor. The values were tested for significant difference with 95% level of confidence. Correlation

Fig. 1. Map of Washington State showing American Viticultural Areas (AVAs) (source: <https://www.washingtonwine.org/>). Number of vineyard blocks and grapevine samples collected from individual AVAs are listed in boxes.

between visual symptoms in red-berried cultivars and the presence of GLRaV-3 and GRBV were examined using PROC FREQ of SAS for Fisher's exact test and Chisq analysis. Correlation between uniplex (separate PCR assays for individual viruses) and multiplex PCR results was analyzed by linear regression in SAS.

Sequence analysis of GRBV isolates. DNA-enriched preparations from GRBV-positive samples were prepared using the cetyl trimethyl ammonium bromide (CTAB) method with slight modifications (Zhang et al. 1998). The primers 2StuF (5'-AGGCCCTTGTCAGTTTGCA TTC-3', corresponding to nucleotides between 809 and 829 in the GRBV genome sequence, accession no. KP221559) and 2Stu R (5'-AGGCCTACTACGAGGCCG-3', corresponding to nucleotides between 814 and 797 in the GRBV genome sequence, accession no. KP221559) were used to amplify the complete genome of GRBV (Poojari et al. 2013). Cloning of amplicons and sequencing of recombinant clones was carried out as described earlier (Poojari et al. 2013). When required, additional clones were sequenced to resolve ambiguities in the sequence. Nucleotide sequences generated for GRBV isolates were edited manually and assembled using BIOEDIT 7.2.5 (Hall 1999). Multiple sequence alignments were made using MUSCLE (Edgar 2004). The alignments were subsequently used to construct a phylogenetic tree using neighbor-joining method (Saito and Nei 1987) in MEGA 7 (Kumar et al. 2016) with 1,000 bootstrap replicates and visualized using Treegraph2 (Stöver and Müller 2010).

Results

The survey revealed that GLRaV-3 was more common than GRBV in Washington State vineyards. Commercial vineyard blocks established between 1990 and 2015 were randomly selected from eight AVAs, representing the major grapevine-growing regions in Washington State. Between the 2014 and 2016 seasons, samples from a total of 2,063 grapevines, composed of 1,938 vines from 18 red-berried cultivars and 125 vines from seven white-berried cultivars, were collected for virus testing (Fig. 1 and Supplementary Table S1). Among the red-berried cultivars, the largest number of samples (927 samples) were collected from cv. Cabernet Sauvignon followed by cv. Merlot (474 samples). Among the different AVAs, the majority of samples were collected from Horse Heaven Hills followed by Yakima Valley, whereas the lowest number of samples were obtained from Lake Chelan (Fig. 1). Of the samples from 2,063 vines tested, 67.77% and 6.01% were positive for GLRaV-3 and GRBV, respectively. In addition, 9.06% of samples tested positive for GLRaV-3 and GRBV, indicating coinfection of both viruses in individual vines. In contrast, 17.16% of samples tested negative for both viruses. These results indicated that significantly higher percentage of vines were infected with GLRaV-3 compared with GRBV alone or as coinfection with GLRaV-3 ($P < 0.0001$).

The occurrence of GLRaV-3 and GRBV as single and coinfection in samples collected from individual AVAs was analyzed to determine possible differences in infection rates within and across AVAs (Fig. 2). Samples positive for GLRaV-3 and GRBV were included in this analysis, while excluding samples negative for both viruses. The results indicated that 64% of samples from Columbia Valley, 61% from Horse Heaven Hills, 59% from Rattlesnake Hills, 78% from Red Mountain, 59% from Wahluke Slope, 66% from Walla Walla Valley, and 60% from Yakima Valley were positive for GLRaV-3 (Fig. 2). Results from samples collected from Lake Chelan AVA was not subjected to statistical analysis due to low sample size. Overall, these results showed that the infection rate of GLRaV-3 alone was significantly high in samples from all AVAs compared with GRBV as single and coinfection with GLRaV-3. However, a comparison of GRBV as single versus coinfection with GLRaV-3 showed differences within and across AVAs. The results shown in Figure 2 indicated that the proportion of vines tested positive only for GRBV were relatively higher than coinfection with GLRaV-3 in the Rattlesnake Hills, Wahluke Slope, and Walla Walla Valley AVAs. Conversely, the proportion of samples that tested positive for both viruses as coinfections was higher than samples tested positive for GRBV alone in the Columbia Valley, Horse Heaven Hills, and Yakima Valley AVAs. In contrast, none of the samples from the Red

Mountain AVA tested positive for both viruses, indicating absence of coinfection of the two viruses in vines tested in this study. Statistical analysis of these results indicated no significant difference between proportion of samples positive only for GRBV and those positive for both GRBV and GLRaV-3. Based on these results, it is reasonable to conclude that there is a greater incidence of GLRaV-3 compared with GRBV in vineyard samples collected from the seven AVAs between the 2014 and 2016 seasons.

Interestingly, 17.16% of samples (354 of 2,063) tested negative for both GLRaV-3 and GRBV. When a subset of these samples showing red leaf symptoms (223 of 354) were tested for GLRaV-1, -2, and -4, the results indicated that samples from 44 vines were positive for GLRaV-4, 10 vines positive for GLRaV-2, and none for GLRaV-1. It should be noted that none of the samples from 354 vines were tested for other viruses and graft-transmissible agents in this study. Nonetheless, these results indicated that not all samples from red-berried cultivars displaying red leaf symptoms were infected with either GLRaVs or GRBV. However, χ^2 test indicated a significant correlation between symptoms and the presence of GLRaV-3 and/or GRBV ($P < 0.05$) in red-berried cultivars with the probability of finding either virus in symptomatic samples at 83%.

The virus indexing results were also analyzed to determine correlation between age of the vineyard block and the presence GLRaV-3 and GRBV as single and coinfections. Results of the analysis showed a significant effect ($P < 0.05$) of the age of vineyard blocks on the occurrence of GRBV than GLRaV-3 (Supplementary Table S2). The data indicated that infection rate of GLRaV-3 was high in samples collected from all vineyard blocks, independent of age of the vines or planting period. In contrast, samples from vines planted after the 2000s had a significantly higher infection rate of GRBV as single virus infection than in samples from vines planted before the 2000s. Similarly, significant difference was observed in GRBV infection rate between single and coinfections in samples from vines planted before the 2000s than in samples from vines planted after the 2000s. Overall, these results suggest that the infection rate of GLRaV-3 was consistently higher in samples of all age groups of vineyard blocks, whereas GRBV infection rate was relatively higher in samples from vineyard blocks planted after the 2000s compared with those planted before the 2000s.

Symptoms of grapevine leafroll and red blotch diseases are highly variable in red-berried cultivars. Typical symptoms of GLD, consisting of reddening or reddish-purple discoloration of leaf tissue between major veins, 'green veins' due to a narrow strip of

Fig. 2. Infection rates of grapevine samples collected from individual American Viticultural Areas (AVAs) during 2014, 2015, and 2016 seasons. Each bar represents mean proportion of samples positive for GLRaV-3 and GRBV as single and coinfections. Samples tested negative for GLRaV-3 and GRBV were excluded from this analysis. AVAs shown are: 1, Columbia Valley; 2, Horse Heaven Hills; 3, Rattlesnake Hills; 4, Red Mountain; 5, Wahluke Slope; 6, Walla Walla Valley; 7, Yakima Valley. Lake Chelan AVA was not included in this analysis due to small number of samples collected. Bars with different letters indicate significant difference in infection rates ($P < 0.05$).

green tissue on both sides of major veins, and downward leaf rolling toward the end of the season (Naidu et al. 2014), were observed in red-berried cultivars (Fig. 3A, I, M and Fig. 4A). In the case of red blotch, red veins on abaxial surface and red blotches or specks on adaxial surface of leaves near harvest, characteristic symptoms of the disease (Poojari et al. 2013; Sudarshana et al. 2015), were observed in several red-berried cultivars (Fig. 3B, J, N and Fig. 4B). In contrast, symptoms of leafroll or red blotch, consisting of mild yellowing of leaves between veins with or without downward rolling of leaf margins (Naidu et al. 2014; Sudarshana et al. 2015), were observed in white-berried cultivars (data not shown). During survey of vineyards, many red-berried cultivars showed red leaf symptoms of various types that did not align with typical symptoms of red blotch and leafroll (Figs. 3 and 4). In some cases, leaves displaying characteristic symptoms of GLD tested positive for GRBV and, conversely, those showing typical symptoms of GRBD tested positive for GLRaV-3 (Fig. 3C-H, K, L, O, P). In addition, leaves of individual vines tested positive for either GLRaV-3 or GRBV showed wide

variation in red leaf symptoms that did not match with typical symptoms of GLD or GRBD (data not shown). In some red-berried cultivars, such as Syrah and Sangiovese, vines tested positive for GRBV showed marginal necrosis and necrotic blotches that coalesced with time resulting in complete necrosis of leaves toward end of the season (Fig. 4C and D).

Multiplex PCR assay for simultaneous detection of GLRaV-3 and GRBV. Instead of testing individual samples in separate PCR assays (uniplex PCR) for GLRaV-3 and GRBV, a multiplex PCR protocol was developed for simultaneous detection of these two viruses in samples collected from individual vines. Initially, the annealing temperature, concentration of primers specific to GLRaV-3, GRBV, and nad5 gene, and component reagents were optimized in comparison with uniplex RT-PCR/PCR assays (data not shown). The optimum reagent concentrations and PCR conditions described in Materials and Methods were subsequently used to test 535 samples (467 samples from nine red-berried cultivars and 68 samples from three white-berried cultivars) for the presence of

Fig. 3. Red leaf symptoms observed in cvs. Cabernet Sauvignon (A-H), Merlot (I-L), and Cabernet franc (M-P). Samples tested positive for GLRaV-3 and GRBV are indicated on top of each column.

GLRaV-3 and GRBV. For validation of these results, the same sample extracts were tested concurrently for GLRaV-3 and GRBV in uniplex PCR assays. In multiplex assays, amplicons of 181 bp specific to *nad5* gene were consistently amplified in all samples, irrespective of whether these samples tested positive or negative for GLRaV-3 and GRBV (Fig. 5). In some samples, a 540-bp fragment specific to GLRaV-3 and in some samples a 285-bp fragment specific to GRBV were amplified. In some samples, DNA fragments specific to GLRaV-3 and GRBV were amplified indicating coinfection of both viruses (Fig. 5). To validate these results, amplicons from representative samples were cloned, sequenced, and the derived sequences compared with corresponding sequences from GenBank to confirm multiplex PCR results (data not shown). The results from testing 535 samples by multiplex PCR showed coinfection of both viruses in 63 samples (12%) and single infection of GLRaV-3 and GRBV

in 347 (65%) and 33 (6%) samples, respectively, and a total of 82 samples (15%) tested negative for both viruses. Overall, multiplex PCR results showed 98.69% correlation with uniplex PCR results, with a correlation coefficient of 0.999.

Phylogenetic analysis revealed the occurrence of genetic variants of GRBV in Washington State vineyards. The complete genome of 42 isolates of GRBV (GenBank accession nos. MF795144 to MF795177) from 17 wine grape cultivars was sequenced (Supplementary Table S3). Phylogenetic analysis using the neighbor-joining method showed that GRBV isolates from Washington State vineyards segregated into two previously defined clades (Krenz et al. 2014). The majority of GRBV isolates (37) belonged to clade II than clade I, with the genetic diversity of virus sequences between clades greater than within clades (data not shown). Based on these results, it can be inferred that genetic variants of GRBV belonging to two phylogenetic clades are present in Washington State vineyards, independent of cultivar and geographic origin. These results are in agreement with previous reports of genetic diversity of GRBV from other grapevine-growing regions (Al Rwahnih et al. 2015; Krenz et al. 2014; Poojari et al. 2017).

Discussion

This study provided, for the first time, information on the relative occurrence of GLRaV-3 and GRBV associated with leafroll and red blotch diseases, respectively, in Washington State vineyards. The data indicated that GLRaV-3 and GRBV are widespread in all AVAs, but the infection rate of GLRaV-3 is much higher than that of GRBV in samples analyzed, independent of geographic origin of samples and cultivars, suggesting that the significance of GLRaV-3 to vineyard sustainability in Washington State is far greater than GRBV. Although this study represents infection rates of the two viruses in symptomatic vines mostly from red-berried cultivars with a good coverage of the wine grape production areas in eastern Washington, additional surveys are needed to obtain comprehensive data on the relative incidence of GLRaV-3 and GRBV in both wine and juice grape cultivars planted in vineyards across the state. It should be noted that there is very little documented spread of GRBV so far in Washington State vineyards and, therefore, the relative importance of GRBV may be much greater in areas where this virus has been documented to spread.

The results of this study raised a fundamental question regarding the symptomatology of GLD and GRBD. Based on diagnostic results, it was apparent that no obvious correlation exists between the spectrum of symptoms observed in red-berried cultivars and the presence of GLRaV-3 or GRBV. A likely explanation could be that the phenotype of GLD and GRBD symptoms observed under field conditions could be variable due to pathogenicity characteristics of viruses present as single and coinfections and expression of symptoms influenced by confounding host-virus interactions and

Fig. 4. Variation in red leaf symptoms observed in cvs. Syrah (A, C), Petite Sirah (aka Durif, B), and Sangiovese (D). Symptomatic leaves on the left tested positive for GRBV and corresponding symptomless samples on right tested negative for GRBV and GLRaV-3.

Fig. 5. Detection of GLRaV-3 and GRBV by multiplex PCR in red-berried cultivars showing red leaf symptoms. PCR products were resolved by electrophoresis in 1.2% agarose gels. Presence of GLRaV-3 and GRBV is shown by arrow head on right, with size of the DNA band (in base pairs) corresponding to each virus shown on left. Lanes 1-4, samples from cv. Merlot; lanes 5-8, samples from cv. Zinfandel; lanes 9-10, samples from Syrah; lanes 11-14, samples from cv. Cabernet Sauvignon; lanes 15-17, samples from Riesling. A DNA band specific to *nad5* amplified in all lanes is shown by arrow head on right and its size shown by arrow head on left. M, 1 Kb plus DNA marker (Invitrogen, U.S.A.). Lanes indicated by '-' and '+' represent negative and positive controls, respectively, from a healthy grapevine (cv. Cabernet Sauvignon) and a grapevine tested positive for GRBV and GLRaV-3.

environmental-specific factors. Although samples were not tested for different viruses known to infect grapevines (Martelli 2017), it is likely that samples collected during this study might be infected with multiple viruses and complex and dynamic interactions between coinfecting viruses and their shared host have contributed to variable symptoms in different red-berried cultivars. Since red-berried cultivars express symptoms of GLD and GRBD in a phenological stage-specific manner and symptoms are highly variable in these cultivars and white-berried cultivars do not exhibit symptoms analogous to red-berried cultivars, virus-specific diagnostic assays are indispensable for reliable detection of these two disparate viruses in vineyards.

Besides GLRaV-3 and GRBV, some samples showing red leaf symptoms tested positive for GLRaV-1, -2, and -4 and other samples were negative for GLRaVs and GRBV. A possible explanation could be that these viruses were present in quantities undetectable by PCR at the time of sampling and/or compounds, especially phenolic and polysaccharides, present in samples collected toward end of the season could have inhibited PCR assays. The nad5 PCRs were all positive in these samples, suggesting that inhibitors were not the reason that some of the samples tested negative for GLRaVs and GRBV. Alternatively, the presence of viruses could have been missed in leaf samples used for testing due to irregular distribution of viruses in individual vines. However, these possibilities were minimized by pooling leaf samples collected from different parts of individual vines and coamplification of a housekeeping gene of host origin (nad5) as an internal control enabled reliable interpretation of negative results. It is plausible that the primers used in this study might not have been able to amplify all variants of GLRaV-3 present in symptomatic samples due to genetic variability of the virus (Maree et al. 2015). Moreover, red-berried cultivars can express red leaf symptoms due to a wide range of confounding biotic and abiotic factors (Gohil et al. 2016; Rayapati et al. 2008). Additional studies are, therefore, needed to further unveil the etiology of red leaf symptoms to distinguish viral symptoms from those induced by biotic and abiotic stress factors in commercial vineyards.

In this study, a multiplex PCR assay was optimized for the simultaneous detection of GLRaV-3 and GRBV in single and coinfections in individual grapevines. The versatility of multiplex PCR for concurrent detection of RNA and DNA viruses infecting plants and animals has been reported previously using conventional and real-time PCR formats (Abarshi et al. 2012; Chang et al. 2007; Ghosh et al. 2008; Otti et al. 2016; Poojari et al. 2016; Xu et al. 2012), demonstrating advantages of multiplex PCR detection of viruses with distinct genomes. The extraction protocol is well suited for preserving integrity of viral genomes in sample extracts and the one step single tube assay successfully amplified viruses with RNA and DNA genomes to provide reliable diagnostic results. Additionally, the single tube-one step multiplex PCR reduced the number of steps involved, thereby minimizing the risk of cross-contamination leading to false positive results and enhancing practical advantages of multiplex PCR detection of GLRaV-3 and GRBV. Including primers for the amplification of mRNA of the mitochondrial nad5 gene further enhanced the validity of multiplex PCR, thereby facilitating proper interpretation of negative results while indexing for GLRaV-3 and GRBV in grapevine samples. In these regards, the multiplex PCR assay developed in this study offers high-throughput and cost-effective diagnostic services for testing large number of samples collected during vineyard surveys.

The consistently high levels of GLRaV-3 in symptomatic samples collected from vineyards planted before and after the 2000s could be due to a combination of compromised planting materials used for planting new vineyards and transmission by grape mealybugs and soft scale insects (Bahder et al. 2013b). Although older vineyard blocks are expected to have higher incidence of GLRaV-3 due to vector-mediated spread in time and space, the results of this targeted study may not reflect the true incidence of the virus in all vineyard blocks planted before the 2000s. The higher incidence of GRBV in samples collected from vineyards planted during the post- than the pre-2000s can be explained by the dissemination of the virus predominantly via contaminated planting materials during the post-

2000 planting boom in Washington State due to the lack of diagnostic methods until the discovery of GRBV in 2012 (Al Rwahnih et al. 2013; Krenz et al. 2012; Poojari et al. 2013). In contrast to GLRaV-3, field spread of GRBV in Washington State vineyards has not been examined, although Virginia Creeper leafhopper was reported to transmit GRBV under greenhouse conditions (Poojari et al. 2013). Recent reports on the spatio-temporal distribution of GRBV-infected vines in a Cabernet franc block in California implicated insect vector(s) in the spread of GRBV (Cieniewicz et al. 2017) and the three-cornered alfalfa treehopper (*Spissistilus festinus* Say.) was shown to transmit GRBV under greenhouse conditions (Bahder et al. 2016). In contrast, there was no evidence yet for the presence of three-cornered alfalfa treehopper in Washington State vineyards. Future studies should involve elucidating the biological and epidemiological aspects of GRBV and GLRaV-3 and the practical significance of genetic variants of these two distinct viruses for strengthening grapevine certification programs and implementing effective disease control strategies in commercial vineyards.

In summary, GLRaV-3 is more widespread than GRBV across vineyards in eastern Washington State and reliable identification of these two viruses is necessary to differentiate GLD and GRBD symptoms from those induced by biotic factors and abiotic stresses in commercial vineyards. A multiplex PCR protocol developed in this study will aid in simultaneous detection of GLRaV-3 and GRBV in grapevine samples collected during vineyard surveys. Overall, these results provided a foundation for a better understanding of epidemiological aspects of GLRaV-3 and GRBV and implementing sustainable strategies to mitigate negative impacts of these two disparate viruses in Washington State vineyards.

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