SYBR Green I-based Real-Time Quantitative Reverse-Transcription PCR for Detection and Discrimination of Grapevine Viruses

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Abstract

A SYBR[®] Green-based real-time quantitative reverse transcription PCR (qRT-PCR) assay in combination with melt curve analysis (MCA) was developed for the detection of nine grapevine viruses. The detection limits for simplex qRT-PCR for all nine grapevine viruses were estimated to be in the range of 214 to 1112 copies of the virus genome. Amplicons with melting temperatures (T_m) separated by at least 2°C in the MCA could differentiate two viruses in the same reaction. Therefore, eight of the nine viruses could be co-diagnosed in five different combinations of duplex assays. Of 305 grape leaf samples from the field or greenhouse, 162 were positive for at least one of the nine grapevine viruses using the duplex qRT-PCR assays. In contrast, only 127 samples were positive using endpoint RT-PCR and PCR assays, indicating the enhanced sensitivity of duplex real-time PCR. In addition, the duplex qRT-PCR assays were used to detect *Grapevine leafroll associated virus 3* (GLRaV-3) in its vector, the grape mealybug (*Pseudococcus maritimus* Ehrhorn), and Grapevine red blotch-associated virus (GRBaV) in Virginia creeper leafhopper (*Erythroneura ziczac* Walsh). The simplex and duplex real-time PCR assays developed in this study can be used to determine transmission efficiencies of insect vectors as well as for rapid and sensitive detection of viruses in infected grapevines.

Keywords

Melt curve analysis, GLRaV, Grapevine viruses, GRBaV, mealybugs, leafhoppers

1. Introduction

About 70 different viruses and virus-like agents have been documented in grapevines (Martelli, 2014). Since these viruses are primarily disseminated via contaminated vegetative cuttings, they pose barriers to establishing new plantings with clean plant materials, thus threatening the long term sustainability of the wine grape industry. Detection of grapevine viruses is complicated by seasonal variations in titer (Wright, 2012), and by the low concentration and uneven distribution of viruses in host tissues (Sharma et al., 2011). Therefore, sensitive and specific methods for the reliable detection of major grapevine-infecting viruses are essential for phytosanitary standards. Furthermore, availability of sensitive and specific diagnostic assays will enable better understanding of compatible virus-grapevine-vector interactions as a precursor to the development of effective management tools. An enzyme-linked immunosorbent assay (ELISA) conventionally is used for the detection of several viruses in grapevines (Gambino et al., 2006). However, accurate detection of viruses by ELISA is hampered in many situations by the lack of virus-specific antibodies, serological variations among virus strains/variants and the flux of grapevine virus titers in their host, as mentioned above. To overcome these limitations and to meet the rising demand for specificity and sensitivity in virus diagnostics, molecular diagnostic techniques such as reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) have been employed for detection and differentiation of grapevine viruses and their variants (Bertolini et al., 2010; Osman et al., 2012).

TaqMan-based qRT-PCR has routinely been used for the detection of viruses in grapevines and in their insect vectors (Bertolini et al., 2010; Čepin et al., 2010; Klaassen et al., 2011; López-Fabuela et al., 2013; Osman et al., 2006; Osman et al., 2007; Osman et al., 2008a; Osman et al., 2008b; Osman et al., 2012). A drawback of TaqMan-based assays is that different probes have to be synthesized for different targets thus increasing assay costs. SYBR[®] Green-based real-time quantitative reverse transcription PCR assays for the simultaneous detection and discrimination of two or more major grapevine viruses in mixed infections would provide an economical alternative. Simultaneous virus detection could be achieved by exploiting differences in the melting temperature (T_m) profiles of the target viral DNA amplicons. Melt curve analysis

(MCA) has been widely used and shown to be extremely reliable for differentiating plant viruses (Bester et al., 2012; Gadiou et al., 2012; Cheng et al., 2013). Another advantage of qRT-PCR over endpoint RT-PCR is that it also can be used to quantify viral copy numbers in a plant or vector sample. Thus, it is possible to investigate the transmission efficiencies of specific viruses in a given host plant insect vector combination.

In this study, five duplex SYBR[®] Green qRT-PCR assays were developed in combination with MCA for reliable and sensitive detection of nine major grapevine viruses, specifically, Grapevine leafroll-associated viruses 1 to 4 (GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4), Grapevine red blotch-associated virus (GRBaV), Grapevine viruses A and B (GVA, GVB), *Grapevine fanleaf virus* (GFLV) and *Tobacco ringspot virus* (TRSV), a virus whose host range includes grapevine. The assays were compared with endpoint RT-PCR using virus isolates collected from grapevines, the grape mealybug (*Pseudococcus maritimus* Ehrhorn.) and the Virginia creeper leafhopper (*Erythroneura ziczac* Walsh.), vectors of GLRaV-3 and GRBaV, respectively (Behder et al., 2013; Poojari et al., 2013).

2.0 Materials and Methods

2.1 Virus Isolates, RNA Extraction and Reverse Transcription

Plant samples that previously tested positive for GLRaV-1, -2, -3, -4, GRBaV, GVA, GVB, GFLV and TRSV were collected from red- and white-fruited wine grape cultivars planted in commercial vineyards in the Yakima Valley of Washington State. The samples were collected during post-véraison stage, a period when the titer of grapevine viruses is known to be optimal in petiole/leaf samples. Total RNA was isolated from petiole samples using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St Louis, Missouri, USA). Total nucleic acids, enriched with DNA, were also extracted separately from grapevine leaves and single mealybug or leafhopper or from cohorts of 20 insects raised on healthy and virus-infected grapevines maintained under greenhouse conditions (leafhoppers) or in commercial vineyards (grape mealybugs) according to Dellaporta et al. (1983). The quality and quantity of nucleic acid preparations was determined using a spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA).

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using random hexamers

and the Transcriptor First Strand cDNA Synthesis Kit, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The cDNA and total nucleic acid preparations were subsequently used for the detection of RNA and DNA viruses, respectively, as described below.

2.2 Simplex and Duplex SYBR[®] Green Real-time qRT-PCR Assays

Species-specific primers were designed based on non-conserved regions in alignments of nucleotide sequences of respective viral isolates using Vector NTI Advance 11 software (Life Technologies, Grand Island, New York, USA). Target regions for primer design were the helicase (HEL) domain of the replicase gene for GLRaV-1, -2, and -3, RNA dependent RNA polymerase (RdRp) for GLRaV-4 and its strains GLRaV-9 and TRSV, and coat protein (CP) for GFLV, GVA, GVB and GRBaV (Table 1; Supplementary Table 1). Initially, experiments were performed to optimize primer concentrations, annealing temperatures, elongation intervals, and PCR reagent concentrations for simplex and duplex qRT-PCR. Primer concentrations ranging from 60nM to 5µM were tested using the simplex assays and recombinant plasmid DNA containing the respective virus-specific target genes. Optimization was assessed based on amplification, a single peak in the melting curve indicating a single amplicon, and visualization of single amplicons of the expected size on an agarose gel. The resulting melting point for individual amplicons shown in Table 2 was used in subsequent experiments described below.

Each simplex PCR reaction was carried out in a total reaction volume of 12µl containing 1X LightCycler[®] 480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN), 60 nM of each primer and 2µl of template cDNA or DNA-enriched total nucleic acids. PCR conditions for simplex PCR were: initial denaturation at 95°C for 3 min, followed by 45 consecutive cycles of 10 s at 95°C, 10 s at 58°C (annealing) and 15 s at 72°C (extension). The duplex PCR reaction was carried out in a total volume of 20µl containing 1X LightCycler[®] 480 SYBR[®] Green I Master, 120 nM each primer and 2µl of template cDNA or DNA-enriched total nucleic acids. PCR conditions for duplex PCR were the same as that for simplex PCR, except that annealing and extension were done for 20 s and 30 s, respectively. All qPCR and qRT-PCR reactions were carried out using the LightCycler 480 System (Roche Applied Science). Non-template (water), healthy and virus-positive controls and replicated reactions were included in compliance with the

Minimum Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Specific amplification of target viral DNA in both simplex and duplex PCR assays was confirmed by cloning PCR amplicons into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequencing the plasmid DNA isolated from recombinant clones using QIAprep spin miniprep kit (Qiagen Inc., Valencia, California, USA). The sequences obtained were confirmed by comparing with corresponding virus sequences available from GenBank.

2.3 Efficiencies and Detection Limits of SYBR® Green Real-Time qRT-PCR Assays

To determine the amplification efficiencies of primers, standard curves were generated from 5-6 serial log dilutions of plasmid DNA of each specific viral fragment, and amplification efficiencies (E) were calculated from $E = 10^{(-1/\text{slope})} - 1$, as previously described (Gutha et al. 2010). Detection sensitivities of simplex assays were determined from the quantification cycle (Cq) of the lowest plasmid dilution that fell within the linear standard curve (Table 3). The coefficients of variation for Tm values obtained for each replicate were calculated and the reproducibility of Tm values for different primer combinations were verified using three replicates of each dilution of recombinant plasmids. All PCR reactions, including positive and negative controls, were carried out in duplicate in adjacent wells of the same PCR plate, and the Tm values represent the averages of duplicate reactions. Criteria for determining optimal assay conditions included (a) generation of a target-specific exponential amplification curve above the threshold level; (b) presence of a single peak in the MCA at the specified Tm (standard deviation $\pm 1^\circ\text{C}$) (c) a lack of amplification signal from non-template and healthy sample controls; and (d) visualization of bands (one band in case of simplex and two in case of duplex PCR) of the predicted sizes using agarose gel electrophoresis.

2.4 Melt Curve Analysis (MCA)

Amplicons were subjected to MCA using the default settings of the LightCycler 480 software (Roche Diagnostics) to identify target amplicons and distinguish them from non-target amplicons. Briefly, amplicons were subjected to gradual heating at the rate of $0.1^\circ\text{C}/\text{second}$ from 40°C to 95°C and melting peaks were calculated by plotting the negative derivative of fluorescence at each temperature increment ($-dF/dT$).

2.5 Assessment of the Assays Using Vineyard Samples

To evaluate the utility of the assays, 305 grapevine samples with single or multiple viral infections were tested for the presence of nine grapevine viruses using five duplex assays in combination with MCA. Grapevine leaf samples were collected during post-varaison stage and total nucleic acids were extracted from petioles as mentioned above. The samples were tested simultaneously using endpoint RT-PCR/PCR assay, depending on the virus genome.

3. Results

3.1 Simplex Real-Time qRT-PCR Assays

Standard curves of all nine grapevine viruses were constructed based on Cq values obtained from a dilution series of each target DNA in grapevine petiole extracts. Linear relationships between Cq values and log plasmid DNA, with regression coefficients (R^2) above 0.98, were obtained for all viruses (Fig. 1). In addition, the efficiencies for all virus-specific primer pairs ranged from 0.98 to 1.14, indicating that the primers were amplifying a majority of the target molecules. Also, the % covariance (0.02 to 0.36) for all nine grapevine viruses (Fig. 1) fall within the acceptable range for qPCR assays. The presence of low levels of fluorescent compounds was evident in assays having >1.0 efficiency, as the efficiencies greater than 100% indicate possible exogenous contaminants or fluorescent compounds that affects the Cq values. The optimal concentration of primers for each of the nine viruses was found to be 60 nM. Amplicon MCA showed a single peak for each set of primers, confirming the homogeneity of reaction products. Furthermore, distinct Tm values were obtained for each of the nine amplicons. Specificity of the primers and their combinations were verified via cloning and sequencing of amplified products (data not shown). The detection range of the target DNA in simplex assays was determined to be 214 - 1112 copies of the virus genome (Table 3) from standard curves based on recombinant plasmid DNA.

3.2 Duplex Real-Time qRT-PCR Assays

Duplex assays were developed for those viruses having amplicons with a minimum difference of 2°C (Table 2). Five combinations of the duplex SYBR[®] Green real-time PCR assay were developed using these criteria. Optimal primer concentrations for the duplex assays were 120 nM

of each primer. The MCA showed two melting peaks corresponding to the expected T_m values of the amplicons (Table 2; Figure 2). For instance, two duplex real-time qRT-PCR assays enabled the simultaneous detection of GLRaV-4 and GLRaV-1 (Fig. 2a) and GLRaV-2 and GLRaV-3 (Fig. 2b), when these viruses are present in co-infected grapevine samples. Another duplex assay enabled the simultaneous detection of GRBaV (a DNA virus) and GLRaV-3 (an RNA virus) in co-infected grapevine samples (Fig. 2c), while concurrent detection of two vitiviruses (Fig. 2d) and two nepoviruses (Fig. 2e) is made possible by the development of two additional duplex assays. These results demonstrated that the duplex SYBR[®] Green qRT-PCR assay is capable of detecting two different grapevine viruses in a single tube PCR assays.

3.3 Validation of Real-Time qRT-PCR Assays Using Field Samples

To evaluate the practical utility and accuracy of the duplex real-time qRT-PCR assay, a total of 305 field and greenhouse-maintained grapevine samples were tested for the presence of the nine viruses. Of these samples, 162 tested positive for at least one of the nine grapevine viruses listed in Table 4. In contrast, only 127 of the 305 samples were virus-positive when diagnosed using endpoint RT-PCR/PCR assays (Table 4). These results indicate that the qRT-PCR is more sensitive than endpoint RT-PCR/PCR for the detection of grapevine viruses from field samples. However, both types of assays resulted in the detection of the same viruses in this subset of 127 samples. Cohorts of 20 grape mealybugs (*Ps. maritimus*) and a corresponding number of leafhoppers (*E. ziczac*) that have been allowed access to the virus by exposure to a grapevine infected with both GLRaV-3 and GRBaV were tested individually for both viruses using the simplex assays. Only GLRaV-3 was detected in 11 out of the 20 mealybugs tested using the assay capable of detecting both GLRaV-3 and GRBaV when present as co-infection (Table 4). As expected, all 20 leafhoppers tested negative for GLRaV-3, whereas all of them were positive for GRBaV (Table 4) using the same assay. It should be noted that the amplification of GRBaV is a consequence of viral DNA present in total RNA used as a template for cDNA synthesis. In contrast, only four of the 20 mealybug samples tested positive for GLRaV-3 by endpoint RT-PCR, although all 20 leafhoppers tested positive for GRBaV by conventional PCR. These results further demonstrate the utility and sensitivity of qRT-PCR assays for detection of grapevine viruses in individual insect vectors. The results also provide further evidence for the specificities

of grape mealybugs and leafhoppers in acquisition of GLRaV-3 and GRBaV, respectively (Poojari et al., 2013).

4. Discussion

Since there is no known source of genetic resistance to grapevine viruses (Oliver and Fuchs, 2011), a key component of viral disease management is the propagation of clean plant materials. Thus, sensitive, reliable and cost-effective assays for the detection of viruses are critical to the sustainable production of grapevines. In this study, simplex and duplex SYBR[®] Green real-time qRT-PCR assays were optimized for the detection, discrimination and quantification of nine grapevine viruses reported from Washington State vineyards. Since the primers for GLRaVs target the viral replicase gene, this method is highly reliable in estimating virus genomic RNA copies in a given sample, with concomitant elimination of the risk of overestimation of viral copy numbers by co-amplification of virus subgenomic RNAs (Pacifico et al., 2011; Sharma and Dasgupta, 2012). In cases where duplex assays were limited by overlapping amplicon melt curves, simplex qPCR assay can be used to estimate the viral titers in case of mixed infections.

SYBR Green-based chemistry provides several advantages to existing TaqMan assays. Dyes that intercalate the minor groove of dsDNA, such as SYBR[®] Green, are suitable for all types of DNA regardless of nucleotide sequence. In contrast, TaqMan-based assays are highly target gene specific. Also, in SYBR[®] Green based assays, specific amplicons can be distinguished from non-target amplicons based on melting temperatures (Sharma and Dasgupta, 2012). In addition, the assays developed in this study provide a rapid and cost-effective alternative to TaqMan qPCR and other real-time PCR chemistries with similar efficiency and accuracy, particularly useful when large numbers of samples are to be analyzed. SYBR[®] Green in combination with MCA has been successfully used to detect single nucleotide polymorphisms in qPCR-generated amplicons (Boyle et al., 2009), and for detection and differentiation of plant viruses (James et al., 2006; Jarosova and Kundu, 2010; Sharma and Dasgupta, 2012). Simultaneous MCA in a closed tube eliminates much of the time required for agarose gel electrophoresis.

In conclusion, the assays developed in this study have potential for virus detection in large scale monitoring programs. The increased sensitivity afforded by qRT-PCR will help in the timely detection of very low virus titers in grapevine and insect vectors, with implications for understanding virus-grapevine-vector interactions. In case of multiple infections, the assays will provide valuable information on virus competition within the same host or host tissue.

Author Contributions

Experiments were conceived and designed by SP, RAN and PO, and performed by SP and OJA. SP and RAN analyzed the data. Reagents, materials and analysis tools were provided by RAN and PO. All co-authors contributed to the writing of the manuscript.

Acknowledgement

This work was supported, in part, by Washington State University's Agricultural Research Center in the College of Agricultural, Human, and Natural Resource Sciences, Wine Advisory Committee of the Washington Wine Commission, USDA-NIFA-Specialty Crop Research Initiative (Award Number 2009-51181-06027), and WSDA Nursery Assessment Funds, and USDA ARS Project Number 5248-22000-012-00D (P.O.). PPNS Number xxxx, Department of Plant Pathology, College of Agricultural, Human, and Natural Resource Sciences Agricultural Research Center Project No. WNPO 0616, Washington State University, Pullman 99164.

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TABLES

Table 1. Primers for simplex and duplex SYBR[®] Green I-based real-time quantitative reverse transcription PCR assays developed in this study.

Primer	Sequence (5' → 3')	Target	Amplicon size (bp)
GLRaV-1F	GTGGAGAGTATGATTCCGTGGTCAC	HEL	267
GLRaV-1R	CACTGGCACGTTAACCTGAGGTCG	HEL	
GLRaV-2F	GATGGTGAAGCGTGACGCTAAGGTG	HEL	241
GLRaV-2R	TATGGTACACATCGTCGTCACCAAGC	HEL	
GLRaV-3F	GTAGTGGACGAAAGGTGGCTCTTC	HEL	170
GLRaV-3R	GACAGACAACACCAATTCCTTCGTC	HEL	
GLRaV-49F	TGAGGTCCCATGTCATGAC	RdRp	457
GLRaV-49R	CCTCAATCTRTTSACCAAYTCAC	RdRp	
TRSV-F*	CAGGGGCGTGAGTGGGGCTCCTG	RdRp	316
TRSV-R*	TCAATACGGTAAGTGCACAC	RdRp	
GFLV-F**	CCAAAGTTGGTTTCCCAAGA	CP	322
GFLV-R**	ACCGGATTGACGTGGGTGAT	CP	
GVA-F***	GACAAATGGCACACTACG	CP	430
GVA-R***	AAGCCTGACCTAGTCATCTTGG	CP	
GVB-F	CCCTACTATCTATAACCCTCTGC	CP	200
GVB-R	GCGAAGGGCTCACACATTTGACG	CP	
GRBaV-F	GCCTTGTCAGTTTGCATTCC	CP	265
GRBaV -R	CTTCCGCTGTTATCACTACC	CP	

GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus. RdRp: RNA dependent RNA polymerase; CP: coat protein; F: Forward primer; R: Reverse primer.

*Fuchs et al., 2010. **Rowhani et al., 1993; ***Minafra and Hadidi, 1994.

Table 2. Average melting temperatures (T_m) of amplicons specific to the grapevine viruses with coefficient of variance (CV%) and standard deviations (SD) for optimized SYBR[®] Green I-based simplex and duplex real-time quantitative reverse transcription PCR assays.

Virus	Simplex assay			Duplex assay		
	Average T _m	SD	CV%	Average T _m	SD	CV%
GLRaV-1	86.57	0.09	0.09	86.30	0.05	0.12
GLRaV-2	83.50	0.09	0.34	83.44	0.08	0.24
GLRaV-3	80.45	0.07	0.20	80.17	0.04	0.08
GLRaV-4	82.66	0.06	0.27	82.66	0.06	0.20
TRSV	80.33	0.07	0.28	80.11	0.04	0.05
GFLV	83.45	0.09	0.33	83.12	0.09	0.16
GVA	85.56	0.08	0.34	85.52	0.05	0.04
GVB	82.90	0.02	0.02	82.87	0.10	0.29
GRBaV	82.72	0.10	0.36	82.72	0.08	0.21

GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus

Table 3. Detection limits of the SYBR[®] Green I-based simplex real-time quantitative reverse transcription PCR assay based on determination of copy numbers for each of the nine grapevine viruses. The detection range determined from standard curves based dilution series of respective recombinant plasmid DNA. The mean C_q values of positive samples were represented with respective copy numbers $\pm$ standard error (SE) of the mean.

Virus	Mean C_q value ($\pm$ SE)	Mean copy number (Calculated)
GLRaV-1	35.26 $\pm$ 28	217
GLRaV-2	34.86 $\pm$ 0.5	1093
GLRaV-3	35.85 $\pm$ 12	1112
GLRaV-4	35.52 $\pm$ 24	510
TRSV	34.51 $\pm$ 18	214
GFLV	35.98 $\pm$ 12	1072
GVA	36.18 $\pm$ 19	1046
GVB	35.65 $\pm$ 07	1104
GRBaV	36.12 $\pm$ 16	1087

GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus

Table 4. Comparison of SYBR® Green I-based real-time quantitative reverse transcription PCR assays with endpoint PCR or reverse transcription-polymerase chain reaction (RT-PCR) for virus detection in field-collected samples from grapevine leaf petiole tissues and insect vectors.

Virus	SYBR based duplex PCR (Virus-positive/total number of samples)			PCR/RT-PCR (Virus-positive/total number of samples)		
	Plant tissue	Mealybugs	Leafhoppers	Plant tissue	Mealybugs	Leafhoppers
GLRaV-1	15/20	0/20	NT	12/20	0/20	NT
GLRaV-2	20/20	0/20	NT	15/20	NT	NT
GLRaV-3	30/30	11/20	0/20	24/30	4/20	0/20
GLRaV-4	20/25	0/20	NT	16/25	0/20	NT
TRSV	10/10	0/20	NT	8/10	0/20	NT
GFLV	4/20	NT	NT	0/20	NT	NT
GVA	5/20	NT	NT	0/20	NT	NT
GVB	18/20	NT	NT	12/20	NT	NT
GRBaV	40/40	0/20	20/20	40/40	0/20	20/20

GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus

Figure Legends

Figure 1. Standard curves for grapevine virus DNAs generated using recombinant plasmid template DNA. DNA is expressed in copies of the target sequence. GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus

Figure 2. Separation of amplicon melting peaks in SYBR[®] Green duplex real-time quantitative reverse transcription PCR assays. Targets are indicated above each peak along with non-target negative controls (NTC). GLRaV: Grapevine leafroll associated virus; TRSV: *Tobacco ringspot virus*; GFLV: *Grapevine fanleaf virus*; GVA: *Grapevine virus A*; GVB: *Grapevine virus B*; GRBaV: Grapevine red blotch-associated virus

Figure 1

GLRaV-1

GLRaV-2

GLRaV-3

GLRaV-4

GRBaV

GVA

GVB

GFLV

ToRSV

Figure -2