

Seasonal dynamics and virus translocation of Grapevine leafroll-associated virus 3 in grapevine cultivars

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Grapevine leafroll-associated virus 3 (GLRaV-3) is associated with grapevine leafroll disease, one of the most economically important viral diseases of grapevines. This disease impacts on both vine health and grape quality; reduction in yield, brix and wine colour are among its detrimental effects. Many methods, including serological and molecular procedures, have been developed for the detection of GLRaV-3; however, there is no PCR-based assay available to quantify virus populations within plant tissues. A real-time RT-PCR assay with TaqMan probe was developed for specific and reliable quantitative detection of GLRaV-3 in infected tissues. The designed primers and probes target the conserved sequence in the RNA-dependent RNA polymerase (RdRp) domain of the viral genome to prevent amplification of most subgenomic and defective RNAs. This protocol was used to examine the seasonal dynamics and translocation of GLRaV-3 in field-grown grapevines. The results showed that the virus spread quickly from trunks to new growing shoots and leaves early in the growing season, and most samples still harboured detectable virus during late summer and autumn. The seasonal progress of one GLRaV-3 isolate was compared in four grapevine cultivars (Chardonnay, Cabernet Sauvignon, Italia and Thompson Seedless). Within cultivars there was little variability in the distribution and translocation of GLRaV-3, except for in Thompson Seedless. This quantitative detection assay will be a valuable tool for GLRaV-3 diagnosis, disease monitoring and population ecology studies.

Keywords: *Ampelovirus*, leafroll disease, real-time RT-PCR, TaqMan, *Vitis vinifera*

Introduction

Grapevine leafroll-associated virus 3 (GLRaV-3), the type member of the genus *Ampelovirus*, family *Closteroviridae* (Fauquet *et al.*, 2005), is associated with grapevine leafroll disease, one of the most economically important viral diseases of grapevines (Martelli & Boudon-Padieu, 2006). Grapevine leafroll disease impacts on both vine health and grape quality. Disease symptoms are most pronounced in autumn with downward rolling of leaf margins, and the development of reddening in interveinal regions of leaves of red cultivars, and leaf chlorosis in white cultivars (Weber *et al.*, 1993). Loss of yield and brix, and reduced wine colour are among the detrimental effects of the disease (Charles *et al.*, 2006). The disease is associated with distinct closteroviruses named GLRaV 1-9, GLRaV-Pr and GLRaV-De (Martelli *et al.*, 2002; Maliogka *et al.*, 2008). Within this virus complex, GLRaV-3 is the predominant species in most vineyards worldwide (Martin *et al.*, 2005; Cabale-

iro & Segura, 2006; Coetzee *et al.*, 2010). This virus has been introduced in most grape-growing areas by exchange and propagation of infected scion and rootstock cuttings, with subsequent local dispersal by vegetative material and insect vectors (Cabaleiro & Segura, 2006; Martelli & Boudon-Padieu, 2006; Tsai *et al.*, 2010).

The transmission ecology of vector-borne plant viruses is mediated by a complex network of interactions that connect host plants, insect vectors and virus. At the level of plant–virus interactions, plant species/cultivar, virus isolate/virulence and virus population may influence disease development. In the case of GLRaV-3, the virus moves from roots and trunks into the expanding shoots and leaves with the onset of growth in spring (Teliz *et al.*, 1987; Monis & Bestwick, 1996). GLRaV-3 is first detected in basal leaves as the season progresses, and soon after flowering the virus translocates to apical leaves, where it is initially found at lower population levels (Teliz *et al.*, 1987).

Although current GLRaV-3 detection protocols are highly sensitive (Osman *et al.*, 2008), there is no PCR-based assay available to quantify virus populations within plant tissues. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) allows

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Published online 23 December 2011

rapid detection and accurate quantification of a target sequence (Mackay *et al.*, 2002; Yeh *et al.*, 2009). The initial objective of this study was to develop a protocol to quantify GLRaV-3 in infected grapevine tissues. The protocol was then used to study virus population fluctuations within plants throughout the growing season. To accurately determine closterovirus accumulation in infected tissues, two main problems need to be overcome: (i) the presence of antisense RNAs, subgenomic RNAs (sgRNAs) and defective RNAs during the virus replication cycle; and (ii) the occurrence of high nucleotide variability among virus isolates. To overcome these problems, primers and probes were designed for GLRaV-3 isolates, and a reliable and sensitive quantitative RT-PCR protocol to determine the actual number of genomic RNA (gRNA) templates in infected grapevine tissues was developed. This protocol was then used to examine seasonal dynamics and translocation of GLRaV-3 in field-grown grapevines.

Materials and methods

Plants and viruses

Leaf samples of virus-infected grapevines (*Vitis vinifera*) were collected from the University of California (UC) Davis Grapevine Virus Collection. The *V. vinifera* cv. Cabernet Sauvignon clone VIS 29 is infected with GLRaV-3, *Grapevine virus B* (GVB) and *Grapevine fleck virus* (GFkV). The VIS 29 clone was from old plantings in the Napa Valley, California. Accession LR101 of *V. vinifera* cv. Italia is infected with a GLRaV-3 variant that belongs to the NY-1 genetic group. Samples from three grapevine cultivars (Chardonnay, Cabernet Sauvignon and Thompson Seedless) infected with the same isolate (LR101) of GLRaV-3 through grafting were also collected from the UC Davis Grapevine Virus Collection.

Plant tissue sampling structure

To monitor the virus translocation and distribution in infected grapevines throughout the growing season, the population of GLRaV-3 in field-collected samples from a virus-infected cv. Cabernet Sauvignon clone VIS 29 was examined in 2007. Leaf petiole samples from the apical, middle and basal portions of shoots were collected monthly from April to October and stored at -80°C until total RNA extraction and virus quantification by real-time RT-PCR assay (described below). Samples were collected monthly from the same infected vines as follows: one petiole from each sampling location (apical, middle and basal) on four shoots from three plants, for a total of 36 petioles per month. In April, only apical and basal petiole samples were collected because the shoots were still very short. Petiole samples were used for RNA extraction and quantitative RT-PCR assay because they generally harbour higher populations of GLRaV-3 than leaf blades (Monis & Bestwick, 1996; Ling *et al.*, 2001). In 2008, the population of GLRaV-3 in the VIS 29 clone was exam-

ined again in May, July and September. A total of 18 leaf petiole samples (three leaf positions \times two selected shoots \times three plants) were collected for virus quantification. To compare differential virus distribution among grapevine cultivars, leaf samples from the apical, middle and basal portions of shoots from four LR101-infected grapevine cultivars (Chardonnay, Cabernet Sauvignon, Italia and Thompson Seedless) were collected on the same days for a total of 18 leaf samples (three leaf positions \times two selected shoots \times three plants) for each cultivar each month.

Total RNA extraction

Leaf petioles were chopped and then stored at -80°C immediately after returning from the UC Davis vineyard. To standardize the protocol, the collected petioles were chopped into small pieces and approximately 100 mg of tissue was kept for RNA extraction. Usually, petioles from middle and basal leaves were heavier than 100 mg, but those from apical leaves did not always reach 100 mg. The actual weight of chopped petioles was recorded and used for later calculation. When extracting total RNA, petioles were flash-frozen in liquid nitrogen and ground thoroughly with a mortar and pestle immediately after nitrogen evaporation. Tissue powders were immediately added to 450 μL QIAGEN buffer RLT, and total RNA was extracted with the RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions with minor modifications. β -mercaptoethanol (1%) and polyvinylpyrrolidone 40 (2.5%) were added to buffer RLT before use. To completely recover all lysate, the mortar was rinsed with another 200 μL buffer RLT after transferring the lysate from the mortar to a 2 mL microcentrifuge tube, and the rinsed RLT was added to the same tube. Subsequently, the combined lysate was transferred to a QIAshredder spin column and centrifuged at 14 000 g for 2 min. The resulting supernatant of the flow-through was mixed with 0.5 volumes of ethanol and then transferred to an RNeasy spin column. After RNA binding to RNeasy membrane silica, the column was washed once with buffer RW1 and twice with buffer RPE. Finally, RNA was eluted with 100 μL of RNase-free water, quantified with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies) and stored at -80°C until subsequent analysis.

TaqMan primer and probe design

The GLRaV-3 population in petiole samples was quantified using a TaqMan one-step RT-PCR assay. Primers and a minor-groove binder (MGB) probe targeting the conserved region of the GLRaV-3 RNA-dependent RNA polymerase (RdRp) domain were designed using the PRIMER EXPRESS software (Applied Biosystems). To design a primer set and probe for most isolates, the RNA sequences of the GLRaV-3 RdRp domain from many geographical locations including Africa, Australia, Asia, Europe, Middle East and the United States were retrieved

from GenBank and aligned by CLUSTALX software (Thompson *et al.*, 1997). Three conserved regions of the GLRaV-3 RdRp domain were chosen for primer and probe design: (i) nt 162–298; (ii) nt 315–457; and (iii) nt 549–653 corresponding to the RdRp domain sequence of GLRaV-3 NY-1 isolate. No qualified primers and probes were found in conserved regions 2 and 3 using the PRIMER EXPRESS software. In conserved region 1, a forward primer (LR3qrtF; 5'-CTTCTACCACGGGATGGACACT-3') and a reverse primer (LR3qrtR; 5'-CATA-GGTGTAGTATTGCCGGATGT-3') were selected (Fig. 1). RT-PCR would yield an amplicon of 83 bp. FAM dye and MGB were chosen as a reporter and a non-fluorescent quencher, respectively, for a TaqMan probe (LR3qrtP; 5'-FAM-CATTGAGGAACAATTTG-MGB-3') (Fig. 1).

Because the primers and probe failed to detect the GLRaV-3 VIS 29 isolate, another set of primers and probe were designed for this exceptional isolate. Conventional RT-PCR was performed using a primer set P3U-2 (5'-GCTCATGGTGAAGCAGAC-3') and P3D (5'-CT-TAGAACAAAAATATGGAGCAG-3') (Turturo *et al.*, 2005). Purified product of the RT-PCR reaction was sequenced directly. A forward primer (LR3qrtF1; 5'-GCTCTTTTACCACGGGATGGA-3') and a reverse primer (LR3qrtR1; 5'-CCCCAAGTTATTCCTCAA-TGTAG-3') were designed from the same region in the GLRaV-3 RdRp domain using the PRIMER EXPRESS software. RT-PCR would yield an amplicon of 62 bp. The new designed TaqMan probe for the VIS 29 isolate was LR3qrtP1 (5'-FAM-CTGCGGAGTTGGC-MGB-3'). BLAST searches against public databases were performed

to confirm that the designed primers only amplified the target sequence in GLRaV-3.

TaqMan one-step RT-PCR assay

Quantitative RT-PCR reactions were performed with the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) according to the manufacturer's instructions. The reactions were performed in Fast Optical 96-well plates on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Fast Optical plates have 30 µL maximum reaction volumes, compared to 100 µL capacity of standard plates, which makes the smaller reaction volume possible. Primer and probe concentrations were optimized to yield the lowest threshold cycle (C_t) and maximum normalized reporter signal (ΔR_n). To prevent imprecise pipetting at small volumes, a reagent cocktail was made as follows: 6 µL of 40 µM forward primer, 12 µL of 50 µM reverse primer, 12 µL of 10 µM TaqMan probe, and 30 µL of 40× Multi-Scribe and RNase Inhibitor Mix (supplied with the kit) were added to 60 µL of RNase-free water to bring the final volume to 120 µL. This reagent cocktail was added to 600 µL of 2× Master Mix (supplied with the kit) to make 720 µL of 'Super Mix'. Single-well reactions were set up as follows: 6 µL of 'Super Mix' and 4 µL of RNA template in a 10 µL reaction. Normally, a 10-fold dilution of total RNA extracted from collected leaf petioles was used as template. The one-step thermal cycling conditions consisted of reverse transcription at 48°C for 30 min, DNA polymerase activation at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Each run (plate) included three replicate reactions each for testing samples, RNA standards (GLRaV-3 transcripts), healthy plant control and non-template control (NTC). A ten-fold serial dilution of *in vitro* transcribed RNA (described below) was used to generate standard curves for the quantification of GLRaV-3 populations in the testing samples. To account for the possibility of any inhibitory effect by substances in grapevine tissues, total RNA extracted from virus-free grapevine tissues was spiked with RNA standards and NTC control. A passive reference dye ROX (supplied with the kit) was incorporated into the RT-PCR reaction in order to normalize the non-PCR-related fluctuations in fluorescence signal.

Data acquisition and analyses were performed with the ABI PRISM Sequence Detection System (SDS) software (Applied Biosystems) using the automatic baseline and threshold features. No amplification was observed with either healthy plant control or non-template control, confirming the specificity of the assay. Regression analysis of a standard curve was used to quantify GLRaV-3 genome equivalent copies in each testing sample.

RNA standard

Partial RdRp domains of GLRaV-3 VIS 29 and LR101 isolates were amplified from infected grapevine total

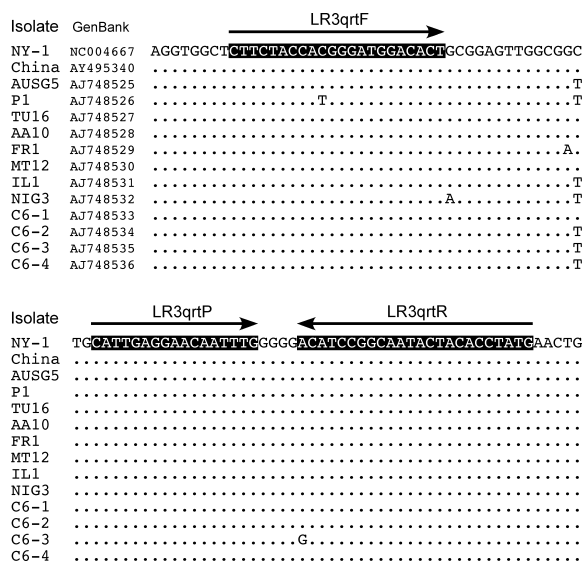


Figure 1 Sequence alignment of Grapevine leafroll-associated virus 3 isolates used to design the primers and TaqMan probe. The forward primer (LR3qrtF), probe (LR3qrtP) and reverse primer (LR3qrtR) are shown boxed. The sequence corresponds to nucleotide positions 7585–7680 of GenBank accession NC004667.

RNA using primer pair T7P3U-2 (5'-TAATACGA CTCACTATAGGG GCTCATGGTGAAAGCAGAC-3') and P3D by the OneStep RT-PCR Kit (QIAGEN). A 5' T7 RNA polymerase binding site (with underline) was incorporated in the forward primer. The reverse transcription was performed at 50°C for 30 min, followed by a PCR activation step at 95°C for 15 min, amplification of 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. The obtained PCR products (653 bp) were purified by QIAquick PCR Purification Kit (QIAGEN) and then used as templates for *in vitro* RNA synthesis. *In vitro* RNA synthesis was performed by MEGAscript T7 Kit (Ambion) following the manufacturer's instructions. RNA transcription was performed at 37°C for 6 h using the T7 RNA polymerase. The transcript was treated with TURBO DNase (supplied with the kit) to remove the template DNA and then recovered by lithium chloride precipitation following the manufacturer's instructions. The concentration was determined using a NanoDrop ND-100 spectrophotometer. The molecular weight of the RNA transcript was calculated by an online tool (<http://www.encorbio.com/protocols/Nuc-MW.htm>; EnCor Biotechnology Inc.), and the Avogadro constant was used to estimate the number of copies of GLRaV-3 RdRp RNA. Ten-fold serial dilutions (10^1 – 10^8 copies) were prepared by mixing the RNA transcript with virus-free grapevine (*V. vinifera* cv. Cabernet Sauvignon) total RNA and used in the quantitative RT-PCR assays to generate a standard curve for GLRaV-3 quantification in testing samples.

The calculation of virus concentration in leaf petiole samples was performed using the following formula: number of genome equivalent copies per mg of host tissue = number of copies ÷ 4 μ L (RNA template) × 100 μ L (RNA extraction) × 10 (dilution factor) ÷ 100 mg (sample weight).

Data analysis of GLRaV-3 populations within plants

For the 2007 study, GLRaV-3 seasonal infection levels in grapevine tissues were evaluated using a linear mixed effects model (Crawley, 2007), in which vine was a random variable, sampling month was a fixed effect nested within vine, sampling site (i.e. basal, middle and apical) was a fixed effect nested within month, and shoot was a random variable nested within site. This structure is necessary to capture appropriately the temporal and spatial repeated measures that were made for each replicate vine. Because middle petiole samples were not collected in April, all April samples were excluded from the analysis. All infection levels were \log_{10} transformed prior to analysis. GLRaV-3 seasonal infection levels in cv. Cabernet Sauvignon grapevines in 2008 were analysed in the same way as for 2007. For the 2008 study, a similar linear mixed effects modelling approach was used, with the same nesting hierarchy, but with the addition of grapevine cultivar as a fixed effect.

Results

Absolute quantification by TaqMan one-step RT-PCR assay

A primer set and TaqMan probe were designed to develop a quantitative RT-PCR protocol to determine GLRaV-3 gRNAs in infected grapevine tissues. To overcome the above-mentioned problems of amplifying non-genomic RNA templates, the RdRp domain was chosen as a target for the quantitative assay. In addition, the RdRp domain is more conserved than other genes in the GLRaV-3 genome (Turturo *et al.*, 2005), so it is easier to design broad-spectrum primers and probes. The RNA sequences of the GLRaV-3 RdRp domain from isolates from various geographical locations (Fig. 1) were aligned to identify conserved regions for primer and probe design. The primers and probe were based on GLRaV-3 RdRp sequences that are available in GenBank and were expected to work for most GLRaV-3 isolates. After preliminary tests, the primers and probe detected the RdRp target of LR101 isolate, but surprisingly they failed to amplify that of the VIS 29 isolate. The RdRp domain of VIS 29 isolate was therefore sequenced (GenBank accession no. HQ711307), and a two-nucleotide difference between the TaqMan probe and VIS 29 RdRp target sequence was identified. In addition, the forward primer had one mismatch, and the reverse primer had two mismatches; thus, another primer set and probe were designed for the VIS 29 isolate.

The detection threshold of the quantitative RT-PCR protocol with both primer and probe sets was 10^2 copies of target sequence in a single reaction (i.e. 4 μ L total RNA contained 10^2 viral gRNA copies), which is similar to quantitative RT-PCR assays for other closteroviruses (Beuve *et al.*, 2007; Ruiz-Ruiz *et al.*, 2007). Because of this detection threshold, all middle and basal samples with undetermined virus populations were assumed to harbour GLRaV-3 in a concentration of 250 genome equivalent copies per mg of host tissue (10^2 copies ÷ 4 μ L × 100 μ L × 10 ÷ 100 mg). Because apical samples often weighed around 50 mg, all apical samples with undetermined virus populations were assumed to harbour GLRaV-3 in a concentration of 500 genome equivalent copies per mg of host tissue (10^2 copies ÷ 4 μ L × 100 μ L × 10 ÷ 50 mg). Therefore, the actual amount of gRNA copies in plant RNA extracts may be slightly overestimated.

Seasonal progress of one GLRaV-3 isolate in cv. Cabernet Sauvignon

The seasonal progress of GLRaV-3 isolate VIS 29 in infected *V. vinifera* cv. Cabernet Sauvignon was examined by TaqMan one-step RT-PCR with monthly collected field samples in 2007. Virus populations varied significantly among sampling months, but there were no significant differences among petioles from apical, middle or basal parts of shoots in 2007 (Table 1). The virus

Table 1 Statistical results from separate linear mixed effects models for *Grapevine leafroll-associated virus 3* isolate VIS 29 infection levels in leaf samples from *Vitis vinifera* cv. Cabernet Sauvignon in 2007 and 2008

Source ^a	2007		2008	
	<i>F</i> _{df, dfe} ^b	<i>P</i>	<i>F</i> _{df, dfe}	<i>P</i>
Month	6.099 _{2,22}	0.0078	11.996 _{2,4}	0.0206
Site	0.610 _{2,66}	0.5465	8.776 _{2,12}	0.0045
Month × Site	6.393 _{4,66}	0.0002	16.885 _{4,12}	<0.0001

^a'Month' denotes an effect of the time when leaf samples were collected, and 'site' denotes sampling location on grapevine shoots.
^b*F* test value, degrees of freedom for the treatment effect, and degrees of freedom for error.

population ranged from below the detection threshold (10^2) to 10^7 genome equivalent copies per mg of host tissue. GLRaV-3 was readily detected in April in approximately 40% of samples. In May, all basal and middle petioles were positive for GLRaV-3, and half of the apical petioles harboured detectable levels of the virus. For basal petioles, the average virus population increased from April to June and reached the highest population in June (1.0×10^6 copies mg^{-1} , Fig. 2). For middle petioles, the average virus population increased from May to June and reached the highest population in June (1.4×10^6 copies mg^{-1} , Fig. 2). For apical petioles, the average virus population increased from April to July and reached the highest population in July (1.3×10^6 copies mg^{-1} , Fig. 2). Independent of petiole location, the maximum virus population was approximately 10^6 copies mg^{-1} . The virus population began to decrease after June or July (Fig. 2). At the end of the growing season, over 90% of samples had a detectable amount of the virus ranging from 3.8×10^3 to 9.1×10^6 copies mg^{-1} . The results show that the GLRaV-3 population and infection

incidence increased rapidly from April to June, and the virus population decreased to a relatively low level (10^4 – 10^5 copies mg^{-1}) for middle and basal petioles after June, although the infection incidence lasted through the growing season.

In 2008, the seasonal progress of GLRaV-3 was examined in the same vines. Virus population varied significantly with sampling month and petioles from apical, middle or basal parts of shoots (Table 1). The results showed a similar trend of virus translocation within plants. Early in the growing season, basal petioles harboured higher virus populations than middle and apical petioles; then the virus population in all petiole locations reached a maximum. Late in the growing season, apical petioles had higher virus populations than middle and basal petioles (Fig. 3). Compared to the 2007 data, the GLRaV-3 population in 2008 decreased to a relatively low level (10^4 – 10^5 copies mg^{-1}) in basal petioles after July but not in middle petioles.

Seasonal progress of one GLRaV-3 isolate in four different cultivars

The seasonal progress of GLRaV-3 isolate LR101 in four infected *V. vinifera* cultivars (Chardonnay, Cabernet Sauvignon, Italia and Thompson Seedless) was also examined in 2008. Virus population varied significantly with sampling month, but there were no significant differences among cultivars or petiole locations, but cultivar/month and month/site interactions were significant (Table 2). Cultivars differed little in the distribution and translocation of GLRaV-3, except for Thompson Seedless. Independent of petiole location and grapevine cultivar (Chardonnay, Cabernet Sauvignon and Italia), the average virus population increased from May to July and then decreased from July to September (Fig. 4), although

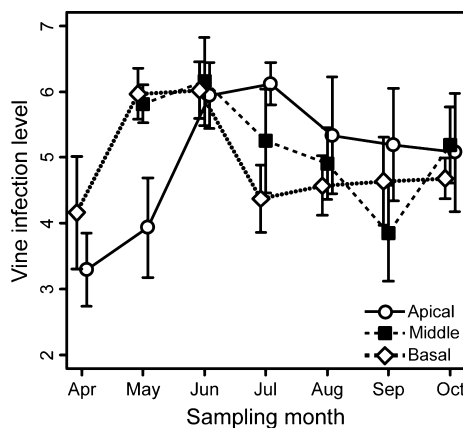


Figure 2 Mean population of *Grapevine leafroll-associated virus 3* isolate VIS 29 [\log_{10} (number of genome copies mg^{-1} host tissue)] in leaf petioles collected from basal, middle and apical sections of cv. Cabernet Sauvignon grapevine shoots between April and October 2007. Points offset slightly for clarity. Vertical bars indicate standard error.

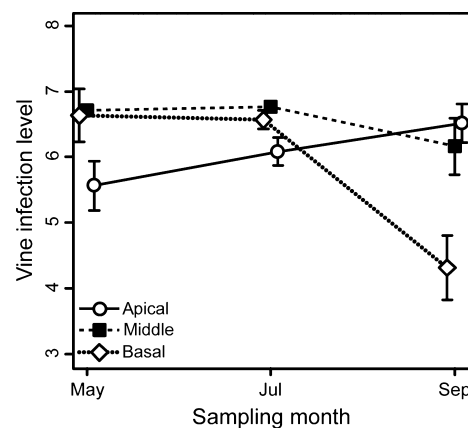


Figure 3 Mean population of *Grapevine leafroll-associated virus 3* isolate VIS 29 [\log_{10} (number of genome copies mg^{-1} host tissue)] in leaf petioles collected from basal, middle and apical sections of cv. Cabernet Sauvignon grapevine shoots in May, July and September 2008. Points offset slightly for clarity. Vertical bars indicate standard error.

Table 2 Statistical results from a linear mixed effects model for *Grapevine leafroll-associated virus 3* isolate LR101 infection levels in leaf samples from four *Vitis vinifera* cultivars in 2008

Source ^a	<i>F</i> _{df, dfe} ^b	<i>P</i>
Month	17.294 _{2,16}	0.0001
Site	0.411 _{2,48}	0.6650
Month × Site	18.841 _{4,48}	<0.0001
Cultivar	2.454 _{3,8}	0.1379
Cultivar × Month	3.766 _{6,16}	0.0157
Cultivar × Site	2.162 _{6,48}	0.0632
Cultivar × Month × Site	1.1626 _{12,48}	0.3360

^a'Month' denotes an effect of the time when leaf samples were collected, 'site' denotes sampling location on grapevine shoots, and 'cultivar' denotes effects of grapevine cultivars.

^b*F* test value, degrees of freedom for the treatment effect, and degrees of freedom for error.

there was an exception for apical leaves in Italia. It is not clear why the average virus population in apical, middle and basal petioles of Thompson Seedless grapevines remained relatively low (10^4 – 10^6 copies mg^{-1}) throughout the growing season (Fig. 4).

A comparison between LR101 and VIS 29 isolates in the same cultivar (Cabernet Sauvignon) indicated a similar trend in virus translocation and distribution throughout the growing season, although LR101 reached higher populations than VIS 29 (*t*-test, *P* < 0.001). In 2008, the

highest average virus population of LR101 isolate was approximately 10^8 copies mg^{-1} in cv. Cabernet Sauvignon, while the VIS 29 isolate was slightly below 10^7 copies mg^{-1} in the same cultivar (Figs 3 and 4). GLRaV-3 isolate VIS 29 is present in the cv. Cabernet Sauvignon host vines with GVB and GFkV while GLRaV-3 isolate LR101 is by itself in the same host vines. Thus, the lower GLRaV-3 populations in VIS 29 isolate may result from the effect of mixed infection with GVB and GFkV. The results may also suggest that these genetically distinct isolates are biologically distinct, and that more work on this aspect needs to be done.

Discussion

Many RT-PCR-based protocols have been developed for the detection of grapevine viruses. Osman *et al.* (2008) reported an assay that detected low populations of GLRaV-3 in plant tissues using TaqMan RT-PCR, but there is no quantitative assay available for this virus so far. A quantitative RT-PCR protocol was developed to determine the absolute amount of GLRaV-3 gRNA in grapevine tissues, and this protocol was used to examine the seasonal translocation and distribution of GLRaV-3 in field-grown grapevines. Importantly, an absolute quantification protocol avoids some of the problems associated with relative quantification methods, which generally assume that transcript level of control genes

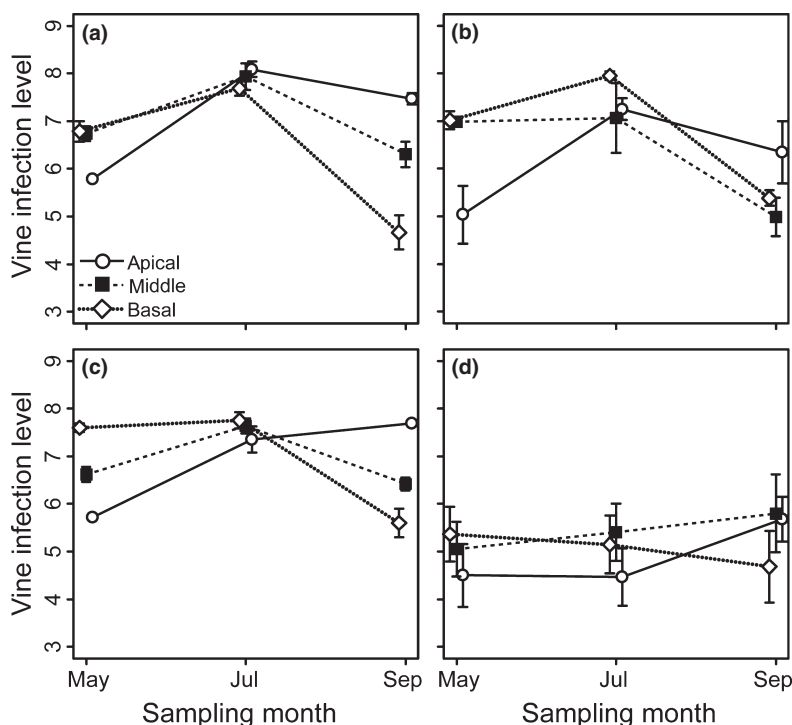


Figure 4 Mean population of *Grapevine leafroll-associated virus 3* isolate LR101 [\log_{10} (number of genome copies mg^{-1} host tissue)] in leaf petioles collected from basal, middle and apical sections of grapevine shoots in May, July and September 2008 for (a) Chardonnay, (b) Cabernet Sauvignon, (c) Italia and (d) Thompson Seedless cultivars. Points offset slightly for clarity. Vertical bars indicate standard error.

keeps constant across seasons, for example. The results showed that the virus spread quickly from trunks to new growing shoots and leaves in the early season; the population reached its highest value within 2 months; yet most samples still harboured detectable virus (10^3 – 10^6 copies mg^{-1}) during late summer and autumn.

The genome structure of GLRaV-3 is similar to that of *Citrus tristeza virus* (CTV), and their replication strategy is assumed to be the same (Dolja *et al.*, 2006). Therefore, at the onset of this study it was assumed that GLRaV-3 infected cells harboured multiple 5' and 3' co-terminal sgRNAs, similar to CTV. During the CTV infection cycle, there are more than 30 different 5' and 3' co-terminal sgRNAs in infected cells (Gowda *et al.*, 2001; Ayllon *et al.*, 2004). Moreover, full length CTV often supports replication of defective RNAs (dRNAs) generated by non-homologous recombination (Mawassi *et al.*, 1995; Che *et al.*, 2002, 2003). To accurately determine the CTV population in virus-infected tissues, finding an appropriate target region for primer design would minimize amplification of non-genomic RNA templates (e.g. sgRNAs and dRNAs). GLRaV-3 open reading frame (ORF) 1a translates to a polyprotein, and ORF 1b (RdRp domain) expresses by +1 ribosomal frameshift (Dolja *et al.*, 2006). A primer set and probe designed from a conserved region in the RdRp domain should enable accurate estimation of genome copies while excluding most non-genomic RNAs. Information about the presence of non-genomic RNAs in infected cells was recently made available for GLRaV-3 (Jarugula *et al.*, 2010; Maree *et al.*, 2010). Combining their discoveries, at least eight putative 3' co-terminal sgRNAs are detected in GLRaV-3 infected grapevine by northern blot hybridization (Jarugula *et al.*, 2010; Maree *et al.*, 2010). The primers and probes designed in this study prevent the amplification of these sgRNAs, therefore accurate estimation of GLRaV-3 copies in grapevine tissues is possible. In addition, the primers and probes were designed based on a conserved region in the RdRp domain so that most GLRaV-3 isolates can be detected. Although the first set of primers and TaqMan probe targeting the RdRp domain worked for the LR101 isolate, it failed to quantify another isolate, VIS 29. The results suggest that the VIS 29 isolate may belong to a different lineage of GLRaV-3, or that the RdRp domain in the VIS 29 isolate is significantly different from that of other isolates whose RdRp sequences were available at the time in GenBank.

Several RT-PCR-based protocols have been developed for quick and sensitive grapevine virus detection (Nakane & Nakano, 2006; Osman & Rowhani, 2006). Real-time RT-PCR increases the sensitivity of virus detection (Osman *et al.*, 2008). Further, TaqMan low-density arrays and oligonucleotide microarrays have recently been introduced as high-throughput approaches for grapevine virus detection (Osman *et al.*, 2008; Engel *et al.*, 2010). Beuve *et al.* (2007) developed new universal primers combined with a real-time RT-PCR assay to estimate GLRaV-2 populations within plant tissues. The assay targeted a sequence located in the 3' end of the virus

genome, so the actual virus population was probably overestimated due to amplification of non-genomic RNAs. Ruiz-Ruiz *et al.* (2007) developed a real-time RT-PCR assay for CTV quantification; the assay targeted the conserved sequences in ORFs 1b and 2 and enabled reliable estimation of CTV genome copies in infected tissues. Overall, results obtained by these authors indicate that the GLRaV-3 quantitative assay in this study reached the same sensitivity as the quantitative RT-PCR assay for other closteroviruses. Saponari *et al.* (2008) employed a TaqMan quantitative RT-PCR protocol to estimate CTV populations in infected plants and viruliferous aphid vectors; however, the assay targeted the coat protein gene, and therefore it overestimated the virus population by amplifying sgRNAs.

Studies have previously reported on the seasonal progress of GLRaV-3 populations in known virus-infected grapevines throughout a growing season. Enzyme-linked immunosorbent assay (ELISA) was used to detect GLRaV-3 in various grapevine tissues over the course of a season (Teliz *et al.*, 1987; Monis & Bestwick, 1996). In addition, Ling *et al.* (2001) compared the effectiveness of ELISA and conventional RT-PCR for detecting GLRaV-3 in field samples. The virus was detected in all tissues including leaves, flowers, roots, fruit, fruit peduncles, tendrils and bark tissue (Teliz *et al.*, 1987; Ling *et al.*, 2001). In spring, GLRaV-3 moved into expanding shoots and was detected in many tissues over the growing season. GLRaV-3 was not detected in leaves until inflorescences were fully developed, and young leaves including the terminal one harboured detectable virus population from berry touch stage until harvest (Teliz *et al.*, 1987). In this study, the same pattern of virus translocation from trunks to basal leaves and then to apical leaves as the season progressed was observed, except that virus populations decreased towards the end of the year. Because Teliz *et al.* (1987) sampled the leaf lamina whereas this study sampled the petioles, the translocation of GLRaV-3 to apical leaves occurred earlier in this study. Furthermore, it should be noted that detection of GLRaV-3 throughout a growing season might be method dependent. Fiore *et al.* (2009), for example, showed that GLRaV-3 detection rate in infected plants remained constant when an ELISA-based method was used, but decreased at the end of the season with RT-PCR. Lastly, some within-treatment variation that was observed may be a consequence of uneven distribution of GLRaV-3 in plants (Monis & Bestwick, 1996), which could be more pronounced under field conditions such as those used here. Some of this variation might have been masked in previous studies by the use of less sensitive quantitative tests such as ELISA.

The virus translocation pattern closely follows the seasonal abundance and distribution of some mealybug vector species, e.g. *Pseudococcus longispinus*, *Ps. viburni* and *Planococcus ficus* in California and possibly elsewhere (Walton *et al.*, 2006; Daane *et al.*, 2008). Mealybug populations increase in spring, and they move from trunks to cordons and leaves after late spring. *Pseudococcus longispinus* and *Ps. viburni* have a relatively narrow

temperature tolerance, whereas *Pl. ficus* infest all parts of the vine year-round (Walton *et al.*, 2006; Daane *et al.*, 2008). As the virus prevalence and population remain high until September, these mealybugs could possibly transmit GLRaV-3 throughout the vegetative growth season.

The quantitative RT-PCR assay developed in this study would reliably estimate the number of GLRaV-3 genomes in infected grapevine tissues. The quantification of GLRaV-3 populations would give new insights into its biology, including the assessment of virus replication in plant tissues, association of symptom severity with specific virus isolates, and monitoring the progress of virus infection during a growing season. This information is also valuable for evaluating plant resistance/tolerance to virus isolates, and for assessing effects of viral load on vector transmission efficiency.

Acknowledgements

The authors thank H.Y. Liu and J. Chau for laboratory assistance, and Foundation Plant Services at the University of California, Davis for access to grapevines used in this study. This research was supported by an award from the United States Department of Agriculture NIFA-SCRI (award no. 2009-51181-06027).

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