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# Identification and distribution of multiple virus infections in Grapevine leafroll diseased vineyards

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**Abstract** A survey of viruses affecting grapevine in the wine regions of the Western Cape Province in South Africa was conducted. The survey determined the relative abundance of five different *Grapevine leafroll-associated virus 3* (GLRaV-3) variants. Virus profiles were also determined for individual vines. A total of 315 plants were sampled and analysed over two growing seasons. Five GLRaV-3 variants were detected as either single or mixed infections, with GLRaV-3 variant groups II and VI being the most prominent as single infections and in combinations with each other and other variants. An analysis of the distribution of variants per region showed that single infections of variant groups II and VI occurred predominantly in certain regions, and were equally distributed in the red and white cultivars studied. The distribution of a recently identified, unclassified variant of GLRaV-3 (represented by isolates GH24 and GTG10) was included in the study. The overall analysis showed that infection with variant groups II and VI were the most abundant among the samples with 49.8 and 47.6 %, respectively, followed by variant group I, variants similar to isolate GH24 and variant group III with 16.2, 13.3 and 2.5 % infection, respectively. Mixed infections, representing 36 virus combinations, were found in 251 plants. The most abundant virus combination was GLRaV-3 with *Grapevine virus E* (GVE), found in 28 % of the plants. GLRaV-3 was the predominant virus detected in the samples with a frequency of 80 % detection, followed by GVE (57.4 %), *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (36.8 %), *Grapevine virus A* (GVA) (19.3 %), *Grapevine virus F* (GVF) (16.25 %), *Grapevine leafroll-associated virus 2* (GLRaV-2) (8.25 %), *Grapevine leafroll-associated virus 1* (GLRaV-1) (1.58 % infection) and *Grapevine leafroll-associated virus 4* (GLRaV-4 like) (0.6 %). Most of the plants tested were infected with multiple viruses. The complexity of virus populations detected in this study, highlights the need for detection methods able to identify all viruses and their variants in vineyards. The information generated in this study will assist in the development of reliable detection assays that will benefit the monitoring of disease spread and aid in the efficient management of Grapevine leafroll disease (GLD).

**Keywords** Grapevine leafroll associated virus 3 · GLRaV-3 · Grapevine infecting viruses · GLRaV-3 variants · Identification · Distribution of viruses

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## Introduction

More than 70 infectious agents have been recorded from grapevines, including 65 viruses (Martelli 2014). This represents the highest number of pathogens found in a single crop. Grapevine leafroll disease (GLD) is one of the major diseases affecting grapevine and occurs in all vine-growing regions of the world. *Grapevine leafroll-associated virus* 3 (GLRaV-3) is known to be closely associated with GLD (Pietersen 2010).

The genetic variability of GLRaV-3 has been studied extensively in recent years from different regions of the world, including: South Africa (Bester et al. 2012a; Jooste et al. 2010, 2011; Goszczynski 2013), USA (Fuchs et al. 2009; Seah et al. 2012; Sharma et al. 2011; Wang et al. 2011), New Zealand (Chooi et al. 2013a, b), Portugal (Gouveia et al. 2011), China (Farooq et al. 2013; Liu et al. 2013) and India (Kumar et al. 2012). The availability of full-length sequences is significantly accelerating the genetic study of GLRaV-3 variants.

To date, six genetic variant groups of GLRaV-3 have been identified world-wide (Maree et al 2008; Jooste et al. 2010; Gouveia et al. 2011; Sharma et al. 2011; Bester et al. 2012a; Chooi et al. 2013a, b). An additional variant group, group VI-like, was identified based on sequence data that showed that the average variation between isolates of groups VI and isolate NZ2 (group VI-like) was 28.3 % (Chooi et al. 2013a; Maree et al. 2013). The nucleotide complexity of variant group VI and VI-like and the authenticity of these variant groups need to be validated as more data becomes available. To date, six full genome sequences, representing four of the genetic variant groups of GLRaV-3, have been published from South African studies, namely: group I (represented by isolate, 621), group II (represented by isolates GP18, 623), group III (represented by isolate PL-20) and group VI (represented by isolates GH11, GH30) (Maree et al. 2008; Jooste et al. 2010; Bester et al. 2012a). Recently, two new GLRaV-3 isolates, GTG10 (Goszczynski 2013) and GH24 (GenBank: KM058745), were identified. Based on sequence comparisons of a 305 nt portion of the HSP70h gene of representatives of the other variants groups, isolates GTG10 and GH24 grouped together (i.e., they are the same genetic variant) but outside of the variant group VI cluster.

The relative abundance of GLRaV-3 variants in GLD plants has been investigated in several studies. In an earlier study the prevalence of variant group II in South African vineyards was shown (Jooste et al. 2011), whereas in China variant group I was found most frequently (Farooq et al. 2013). A higher relative abundance of variant groups I and II was identified in Portuguese vineyards (Gouveia et al. 2009) while in New Zealand group I and VI-like (similar to isolate NZ2) were prevalent in germplasm and in a commercial vineyard. Variant groups I and III were the most frequently detected in a study of Napa Valley vineyards (Sharma et al. 2011). Here, we have added information on the distribution of GLRaV-3 variants in South African vineyards through an extensive survey.

In addition to the GLRaV-3 variant status studies, surveys of GLD associated viruses (GLRaVs) have been conducted in many parts of the world, for example in the USA, New York (Fuchs et al. 2009), California (Sharma et al. 2011), Oregon and Washington (Martin et al. 2005); Tunisia (Mahfoudhi et al. 2008); Chile (Fiore et al. 2008); China (Li et al. 2013) and Turkey (Akbas et al. 2007). These surveys found that mixed viral infections in single plants are common.

In this study we focused on GLD symptomatic plants collected across the main wine-producing regions in South Africa. The composition of virus populations in GLD plants and the relative abundance of GLRaV-3 variants was determined and will be discussed.

## Methods

### Survey and sampling

The survey was conducted in the wine-growing regions of the Western Cape Province, South Africa. The samples were collected from three regions: the Coastal-, Cape South coast- and Breede river valley regions. A total of 315 (171 red cultivar and 144 white cultivar) grapevines were sampled randomly from 29 farms in 2012 and 2013. Most of the collections were done in the Coastal region that included farms in the Darling, Klein Karoo, Paarl, Stellenbosch, Swartland, Tulbagh and Wellington districts. Sampling in the Breede river valley included the Breedekloof and Worcester farms and the Cape south coast sampling were done in the Walker Bay district. A selection of 15 different red cultivars and 10 different white cultivars were sampled that included the economically important cultivars Chardonnay, Sauvignon blanc, Cabernet sauvignon, Merlot and Shiraz. The vineyards selected for the study were all

previously used for propagation material in the grapevine industry, but lost their “mother block” status in the 2008/2009 growing season (for plants selected for the 2012 survey) and in the 2010/2011 growing season (for plants selected for the 2013 survey), due to a GLD relative abundance of more than 3 %. These vineyards were selected to collect data for newly infected plants, assuming that the infection was transmitted from surrounding plants. Petioles and canes from four infected plants were randomly sampled in each vineyard. In red cultivars, GLD symptoms were easily identifiable and sampling was done to include a range of leafroll symptoms. In white cultivars, GLD symptoms were not always distinctive on the plants, except for Chardonnay plants where clear downward rolling of leaves was observed. In white cultivar blocks sampling was done randomly and in some cases based on abnormalities, i.e., yellowing, of plants.

GLRaV-3 variant status

Petioles and phloem scrapings were stored at −80 °C and total RNA extracted from 0.2 g tissue using a modified CTAB (2 % CTAB, 2.5 % PVP-40, 100 mM Tris–HCl pH8, 2M NaCl, 25 mM EDTA pH8 and 3 % β-mercaptoethanol) method (White et al. 2008). Total RNA quality and integrity was evaluated spectrophotometrically (Nanodrop 1000) and with gel electrophoresis (1 % Agarose-TAE).

To differentiate between GLRaV-3 variants in plants and to establish the distribution of single- and mixed infections, a one-step, real-time reverse transcription PCR high-resolution melting curve assay (RT-PCR-HRM) was used (Bester et al 2012b). One hundred to two hundred nanograms of purified total RNA was used as template in the RT-PCR-HRM assay that was performed on a Qiagen Rotor-Gene Q instrument. An RT-PCR assay was developed and used for the specific detection of the GLRaV-3 variant similar to isolate GH24 (GenBank: KM058745).

In order to perform the variant identification, the confidence interval method described in Bester et al. (2012b) was applied, except for the variant group I interval. By including more data points from the 2013 survey this confidence interval was refined. All variant group I infections from 2013 were confirmed by screening the samples with the multiplex RT-PCR described in Bester et al. (2012b). The melting temperature of the confirmed variant group I infections could as a result be added to the existing melting curve data points from Bester et al. 2012b. New variant group I confidence intervals were calculated for both LR3.HRM4 and LR3.HRM6 primer pairs using the additional data points.

Virus populations in leafroll affected plants

*RT-PCR diagnostic*

Primers were either designed through the selection of a conserved region of multiple aligned sequences (CLC Main Workbench V.6.8.4) extracted from GenBank, or selected from publications and databases (Online resource 1). Positive controls for *Grapevine virus F* (GVF), *Grapevine leafroll-associated virus 1* (GLRaV-1), 2 (GLRaV-2), 3 (GLRaV-3), *Grapevine leafroll-associated virus* 4 -like (GLRaV-4), and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) were obtained from the collection at ARC-Plant Protection Research Institute, Pretoria, South Africa. GLRaV-3 isolate GH24, as well as positive controls for *Grapevine virus A* (GVA) and *Grapevine virus E* (GVE) were obtained from the plant collection at the Department of Genetics, Stellenbosch University, South Africa. Two hundred milligrams of each sample was pulverised in a mortar and pestle using liquid nitrogen, and total RNA extracted following an adapted CTAB method (White et al. 2008).

Complementary DNA was synthesised by adding 200 ng of total RNA of each sample to 0.3 μl of random hexamers (Promega) and incubating for 5 min at 65 °C before placing on ice for 2 min to complete the primer annealing reaction. A RT mixture containing 5× AMV RT Buffer (Thermo Scientific), 10 U AMV reverse transcriptase (Thermo Scientific), 10 mM dNTPs (Thermo Scientific) and $dH_2O$ was then added and incubated for 60 min at 48 °C to produce cDNA.

A PCR mixture containing: 2× KAPA Taq buffer A (KAPA Biosystems), 2.5 μM dNTP’s (Thermo Scientific), 5 μM forward primer (Integrated DNA Technologies), 5 μM reverse primer (Integrated DNA Technologies), 2× Cresol Loading dye (30 % sucrose; 125 mg cresol red dye), 1 U KAPA Taq DNA polymerase (KAPA Biosystems), 2 μl cDNA and $dH_2O$, was used to detect each of the viruses in the respective samples. The primer pairs had varying PCR cycle conditions (Online resource 2); all included an initial

denaturation step at 94 °C for 3 min and a final extension step at 72 °C for 7 min.

Statistical analysis of data

The categorical data was summarised in one-way and two-way classifications. For the one-way classifications a chi-square ($\chi^2$) one-sample test was performed to test for significant differences between the proportions. For the two-way classifications a test for a row X column (RxC) table was performed to test for similar patterns or independence (Snedecor and Cochran 1980; Siegel 1956). A $\chi^2$ test was considered significant at the 5 % level if the p-value was less than or equal to 0.05. Data analysis was performed with SAS version 9.3 statistical software (SAS 2012).

## Results

GLRaV-3 variant status

Initial screening of the samples detected the presence of four GLRaV-3 variant groups: I, II, III and VI. Results from the additional RT-PCR assay also confirmed the presence of the new variant, similar to isolate GH24. Positive isolates hereafter were referred to as GH24-like since no variant group has been determined.

Adjusted HRM confidence intervals

Twenty-one plants were identified with the RT-PCR-HRM assay to be potentially infected with only GLRaV-3 variant group I. The multiplex RT-PCR confirmed 15 of these variant group I infections. It was previously reported that the multiplex RT-PCR is less sensitive than the RT-PCR HRM (Bester et al. 2012b). More than one melting point temperature per sample was generated due to duplex reactions. As a result an additional 29 (LR3.HRM4) and 36 (LR3.HRM6) melting curve data points were generated. After re-calculation of the 95 % melting point confidence interval using the 2.5 and 97.5 % percentiles the confidence interval for variant group I was adjusted. The confidence interval for LR3.HRM4 was re-calculated as 83.22 to 84.05 °C and for LR3.HRM6 as 84.82 to 85.90 °C (Online resource 3).

Distribution of GLRaV-3 variants in vineyards

Single and mixed variant infections were detected in the 315 plants screened. The significance of the chi-square test will be indicated in the results description.

*Single variant infections*

One hundred nineteen of the 315 tested plants had single variant infections. Variant groups II and VI were found to be the most prevalent, occurring at frequencies of 47.06 and 37.82 %, respectively. Single infections of group I and GH24-like were recorded to be 6.72 and 7.56 %, respectively ($\chi^2_{(df=4)}=103.98$ $P<0.001$). The distribution of single variant infections was detected in all the districts (Fig. 1a). Variant group II single infections occurred predominantly in the Coastal regions including Stellenbosch, Swartland, Tulbagh, Wellington, Paarl, and Darling. In the Breede River Valley region variant group VI was slightly dominating in the Breedekloof district, while in the Worcester district only one plant with a variant group VI infection was detected. In the Cape South Coast region, the Walker Bay district, variant group VI was the only single variant infection detected in five plants ($\chi^2_{(df=9)}=113.69$ $P<0.001$) (Fig. 1a).

*Mixed variant infections*

As expected, a high number of plants tested positive for mixed GLRaV-3 variant infections. Fourteen variant combinations were detected in 130 plants. In total, 56.25 % of plants collected in the Breede river valley region were infected with multiple variants. In the Cape south coast region 36.36 % of plants had multiple variant infections and in the Coastal region 34.51 % of plants were mixed infected ($\chi^2_{(df=2)}=12.812$ $P=0.002$). The proportion of mixed variant infections was more than 20 % higher in the Breede river valley region. The variant group II/VI combination was the most frequent combination, detected in 43 % of the infected plants. Nine percent of the plants showed mixed infections of variant groups I/II/VI, I/VI and the II/GH24-like combination. The third most frequent variant combination was the II/VI/GH24-like combination, occurring in 6.92 % of the plants ($\chi^2_{(df=13)}=275.57$ $P<0.001$) (Fig. 1b).

The total distribution of GLRaV-3 variants in red and white cultivars were analysed in the three regions

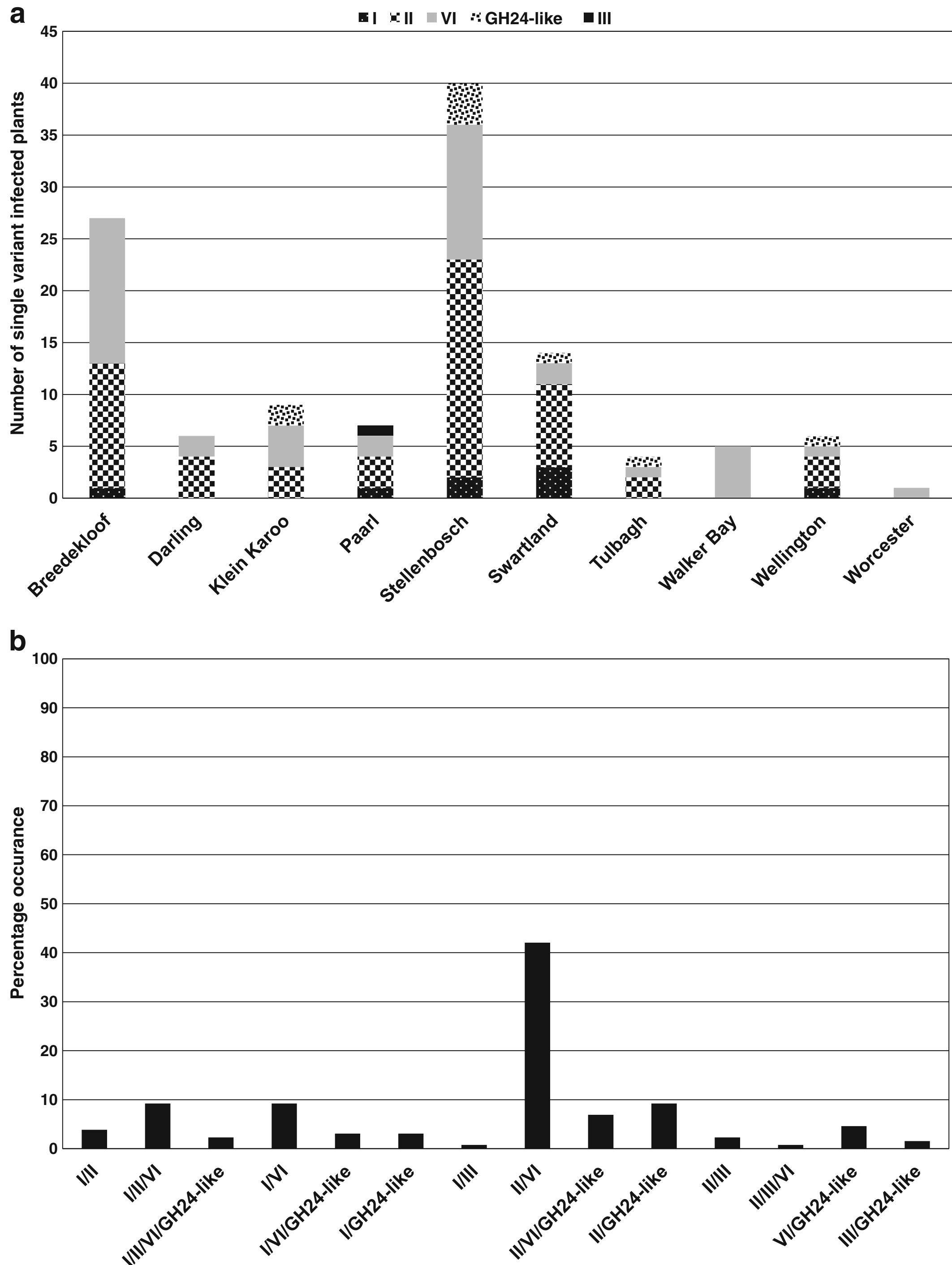


**Fig. 1** Distribution of single variant infections per district (**a**); percentage occurrence of 14 mixed variant combinations in vineyards with variant groups II/VI dominating (**b**)

(Fig. 2a). Variant of group VI occurred dominantly in the Walker Bay district in the Cape south coast region (Fig. 2a) as well as in the Breede river valley region. Variant group II dominated in the Coastal region. No difference between the infection of GLRaV-3 variants in red and white cultivars was detected ($\chi^2_{(df=4)}$=1.38

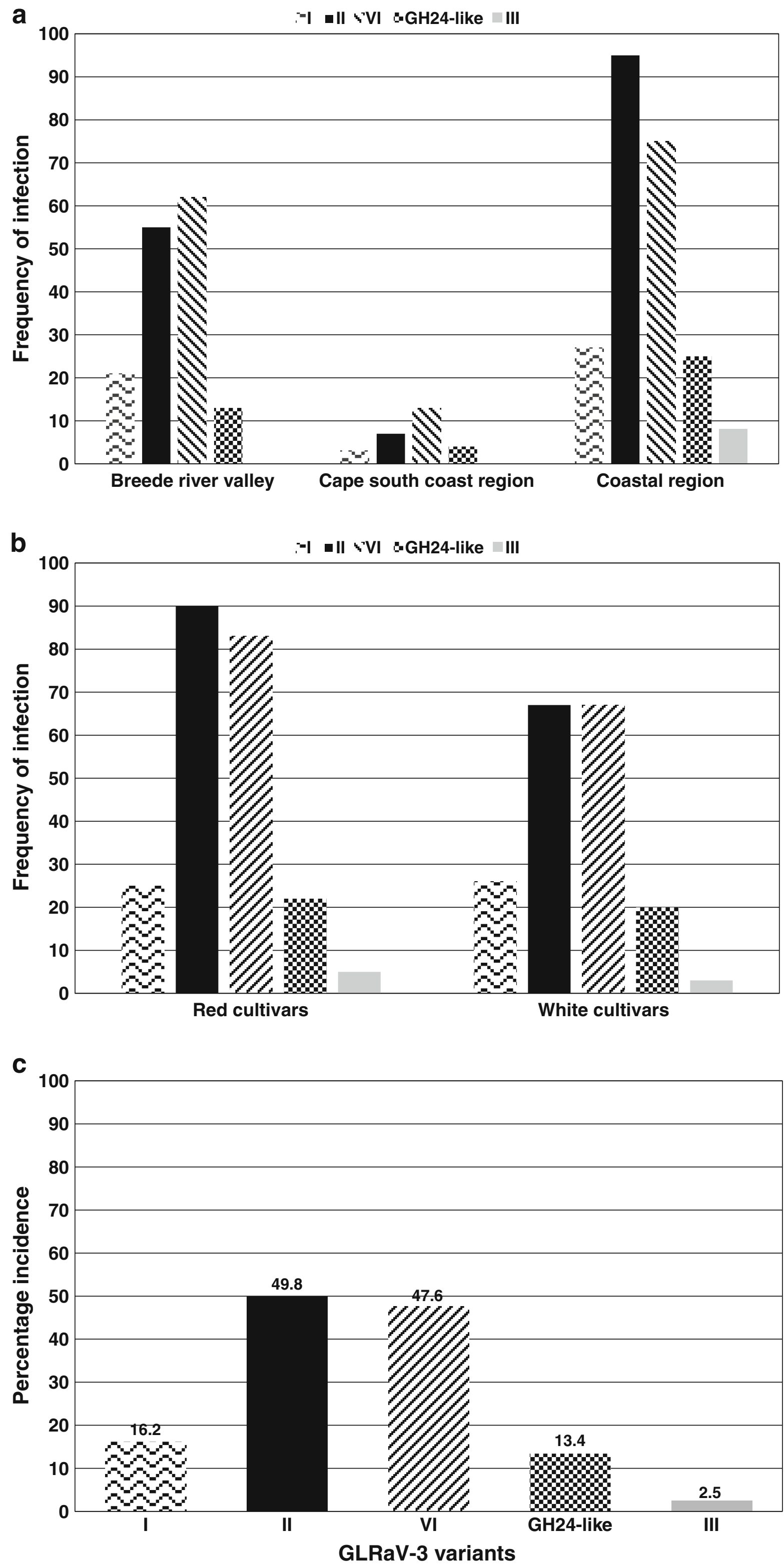


**Fig. 2** The total GLRaV-3 variant distribution per region (**a**); the total GLRaV-3 distribution in white and red cultivars (**b**) and the overall GLRaV-3 variant percentage infection detected in vineyards (**c**)

$P$=0.847) (Fig. 2b). The newly identified GH24-like variant was detected in all the regions and had a high infection percentage in white cultivars in the South coast region. In the Coastal region all five variants were detected, including variant group III from Paarl district. The overall percentage infection of variants are seen in Fig. 2c. Mixed variant infections were detected in all the cultivars in this study.

Virus populations in leafroll-affected plants

*Distribution of other Clostero-, Ampelo-, Viti- and Fovea- grapevine viruses in vineyards*

Virus-specific primers with appropriate positive controls were used in RT-PCR to detect GVA, GVE, GVF, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4–like viruses and GRSPaV. There were 50 plants with single virus infections of GLRaV-2, GLRaV-3, GLRaV-4, GVE, GVF, and GRSPaV. GLRaV-3 was found as a single infection in 29 of these plants and GRSPaV was found as a single infection in 13 plants ($\chi^2_{(df=5)}$=74.5 $P$<0.001).

Most of the plants tested were infected with multiple viruses. Mixed virus infections were found in 251 plants representing 36 combinations of eight grapevine viruses ($\chi^2_{(df=35)}$=780.4 $P$<0.001), indicating that proportions are significantly different. A summary of the mixed virus combinations can be seen in Table 1. The most prevalent mixed virus infection, GLRaV-3/GVE, was detected in almost 28 % of mixed infected plants. The next most prevalent mixed virus combinations were GLRaV-3/GVE/GRSPaV, GLRaV-3/GRSPaV and GLRaV-3/GVA that occurred between 8 and 10 % in plants.

The distribution of these viruses per region are shown in Fig. 3. GLRaV-3 was detected predominantly in all three regions. Other Closteroviruses, GLRaV-1, GLRaV-2 and GLRaV-4-like viruses, were detected in low frequencies in all regions, except GLRaV-2 detected in 19 plants in the Coastal region. The Foveavirus, GRSPaV, was detected between 16 and 22 % in all regions in both red and white cultivar plants. The Vitiviruses screened for, GVA, GVE, and GVF, were present in all regions. GVE was found to be the most widely spread Vitivirus in all regions. The recently discovered GVF virus (Al Rwahnih et al. 2012) was detected in all three regions. The virus distribution was independent of region, meaning the virus distribution of the different viruses in the three regions followed the same pattern ($\chi^2_{(df=14)}$=22.56 $P$=0.0678).

Data from ten districts within the three regions was analysed to determine the prevalence of virus spread per district (Table 2). The majority of positive plants were detected in the Breedekloof and Stellenbosch districts. GLRaV-3 was the most prevalent in all the districts except in the Tulbagh and Walker Bay districts where GVE occurred predominantly. No direct correlation could be made between the plant cultivar and the prevalence of a virus occurring in a certain district. GVE was the prevalent Vitivirus detected in eight of the districts as seen in Table 2.

The total distribution of viruses is summarised in Fig. 4 ($\chi^2_{(df=7)}$=795.12 $P$<0.001). GLRaV-3 was predominantly found in the plants tested in the study, followed by GVE and GRSPaV.

*Viruses detected in different cultivars*

The distribution of the different viruses per cultivar was analysed. Samples were collected from vineyards planted with the following red and white cultivars, respectively: Cabernet sauvignon, Merlot, Shiraz, Chardonnay, Sauvignon blanc, Chenin blanc and Viognier. In Table 3, the frequencies of infection of different viruses per cultivar are shown. GLRaV-3 infected all the cultivars sampled during this study. The other GLRaVs, which were detected in lower frequencies, were detected in white and red cultivar plants and no prevalence to infection of a specific cultivar were seen. The Vitiviruses (GVA, GVE and GVF) and Foveavirus (GRSPaV) were detected widely in most cultivars sampled.

## Discussion

In this study, the distribution of GLRaV-3 variants and other grapevine infecting viruses are presented. We confirmed that GLRaV-3 is the predominant virus in South African vineyards associated with plants showing GLD symptoms. We targeted GLD-affected plants based on symptom expression; however, not all grapevines displayed clear symptomatology, especially in the case of certain white cultivars. It is important to be able to detect all GLRaV-3 variants and other viruses in plants, especially those plants not showing leafroll symptoms. Undetected positive plants are a source of infection and

**Table 1** Mixed virus infections detected in 270 plants of the survey

| Mixed virus combination | Number of plants infected | % infection |
|---|---|---|
| GLRaV-1/GLRaV-3/GRSPaV | 2 | 0.8 |
| GLRaV-2/GLRaV-3 | 3 | 1.2 |
| GLRaV-2/GLRaV-3/GRSPaV | 1 | 0.4 |
| GLRaV-3/GRSPaV | 20 | 7.97 |
| GVA/GLRaV-3 | 21 | 8.37 |
| GVA/GLRaV-3/GLRaV-4 | 1 | 0.4 |
| GVA/GLRaV-3/GRSPaV | 5 | 1.99 |
| GVA/GVE/GLRaV-3 | 14 | 5.58 |
| GVA/GVE/GLRaV-3/GRSPaV | 8 | 3.19 |
| GVA/GVE/GVF | 2 | 0.8 |
| GVA/GVE/GVF/GLRaV-2/GLRaV-3 | 1 | 0.4 |
| GVA/GVE/GVF/GLRaV-2/GLRaV-3/GRSPaV | 1 | 0.4 |
| GVA/GVE/GVF/GLRaV-3 | 4 | 1.59 |
| GVA/GVE/GRSPaV | 1 | 0.4 |
| GVA/GVF/GLRaV-3 | 2 | 0.8 |
| GVA/GVF/GLRaV-3/GRSPaV | 1 | 0.4 |
| GVE/GLRaV-1 | 2 | 0.8 |
| GVE/GLRaV-2 | 2 | 0.8 |
| GVE/GLRaV-2 /GLRaV-3 | 5 | 1.99 |
| GVE/GLRaV-2/GLRaV-3/GRSPaV | 5 | 1.99 |
| GVE/GLRaV-2/GRSPaV | 2 | 0.8 |
| GVE/GLRaV-3 | 70 | 27.89 |
| GVE/GLRaV-3/GRSPaV | 26 | 10.36 |
| GVE/GVF | 1 | 0.4 |
| GVE/GVF/GLRaV-1/GLRaV-3 | 1 | 0.4 |
| GVE/GVF/GLRaV-2/GLRaV-3 | 2 | 0.8 |
| GVE/GVF/GLRaV-2/GRSPaV | 1 | 0.4 |
| GVE/GVF/GLRaV-3 | 8 | 3.19 |
| GVE/GVF/GLRaV-3/GRSPaV | 6 | 2.39 |
| GVE/GVF/GRSPaV | 1 | 0.4 |
| GVE/GRSPaV | 13 | 5.18 |
| GVF/GLRaV-2/GLRaV-3 | 1 | 0.4 |
| GVF/GLRaV-2/GLRaV-3/GRSPaV | 1 | 0.4 |
| GVF/GLRaV-3 | 8 | 3.19 |
| GVF/GLRaV-3/GRSPaV | 5 | 1.99 |
| GVF/GRSPaV | 4 | 1.59 |

The four main virus combinations are highlighted

spread of virus if not identified and eliminated. Overall, in comparison to other Clostero-, Viti-, and Fovea viruses, GLRaV-3 was detected in 80 % of the surveyed plants. In a similar study conducted in China, GLRaV-3 was also shown to be the predominant virus associated with GLD in Chinese vineyards and occurred in 100 % of the tested plants (Liu et al. 2013).

Detection methods were developed to test for five GLRaV-3 variant groups. Four variants groups, I, II, III, and VI were detected in the one step RT-PCR-HRM assay. Another variant, similar to isolates GTG10 (Goszczynski 2013) and GH24 (GH24-like), was detected with a specific RT-PCR. Single and mixed variant infections were detected in plants showing GLD symptoms. Plants with single infections were detected for all variant groups with group II and VI being the most prevalent. A clear regional distribution of plants with single infected variants was seen with variant group II dominating in the Darling, Paarl, Stellenbosch, Swartland, Tulbagh and Wellington districts. Variant

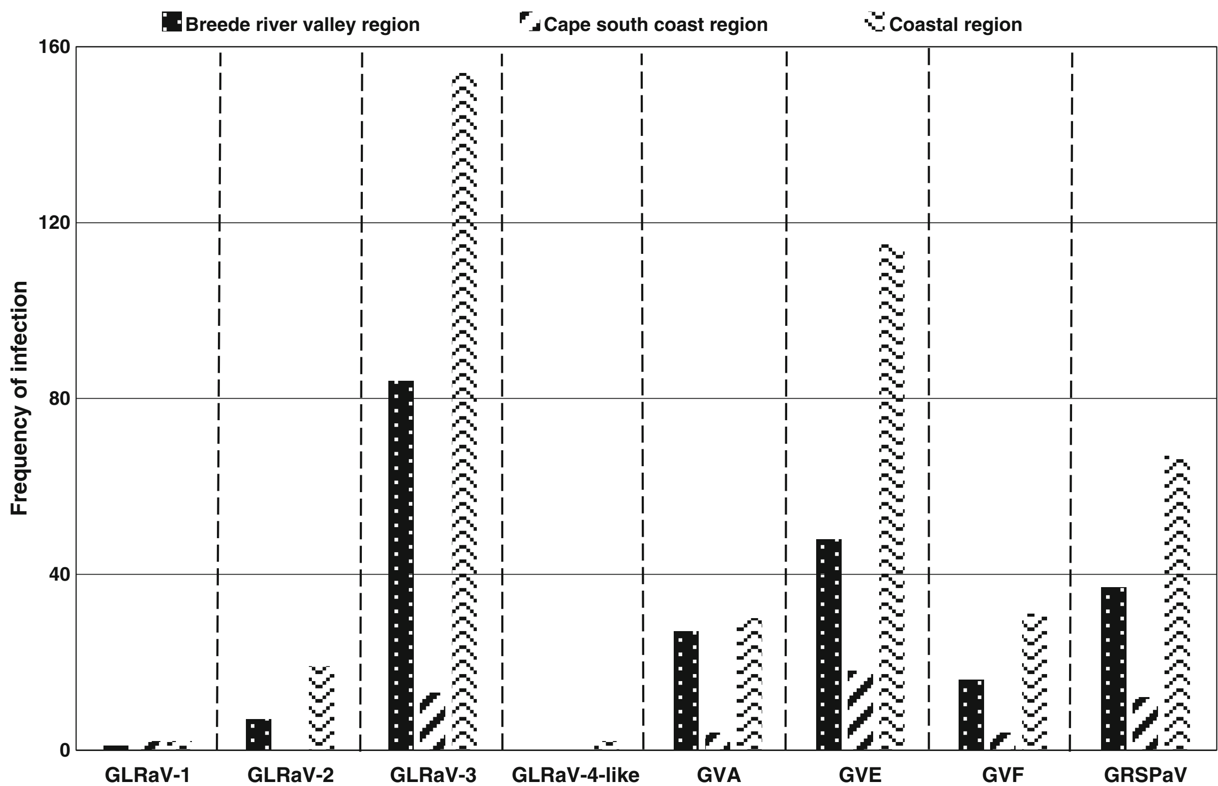


**Fig. 3** Distribution of viruses in three regions of the Western Cape including the Breede river valley region, Cape south coast region and the Coastal region ($\chi^2_{(df=14)}$=22.56 $P$=0.0678)

group VI was detected prevalently in the Breede river valley region including Breedekloof and Worcester districts as well as in the Cape south coast region (Fig. 2a). The overall distribution of the different GLRV-3 variants showed similar distribution in red and white cultivars as shown in Fig. 2b. The study highlighted the importance of being able to test for viruses in white cultivars. White cultivars harbour a wide range of viruses although symptoms are not expressed on plants.

The detection of plants with mixed variant infections was expected. A total of 14 mixed variant combinations were detected in 130 of the tested

**Table 2** Number of positive samples for each virus per district

| | GLRaV-1 | GLRaV-2 | GLRaV-3 | GLRaV-4 | GVA | GVE | GVF | GRSPaV | *N* |
|---|---|---|---|---|---|---|---|---|---|
| Breedekloof | 1 | 7 | 77 | 0 | 27 | 43 | 16 | 33 | 204 |
| Darling | 0 | 0 | 8 | 0 | 3 | 3 | 4 | 3 | 21 |
| Klein Karoo | 1 | 0 | 19 | 0 | 3 | 11 | 4 | 14 | 52 |
| Paarl | 0 | 2 | 15 | 0 | 4 | 11 | 3 | 5 | 40 |
| Stellenbosch | 0 | 13 | 63 | 1 | 10 | 55 | 8 | 23 | 173 |
| Swartland | 0 | 2 | 27 | 1 | 10 | 19 | 7 | 14 | 80 |
| Tulbagh | 0 | 0 | 6 | 0 | 0 | 7 | 1 | 4 | 18 |
| Walker Bay | 2 | 0 | 13 | 0 | 4 | 18 | 4 | 12 | 53 |
| Wellington | 1 | 2 | 16 | 0 | 0 | 9 | 4 | 4 | 36 |
| Worcester | 0 | 0 | 7 | 0 | 0 | 5 | 0 | 4 | 16 |

*N* = Total number of positive samples per district

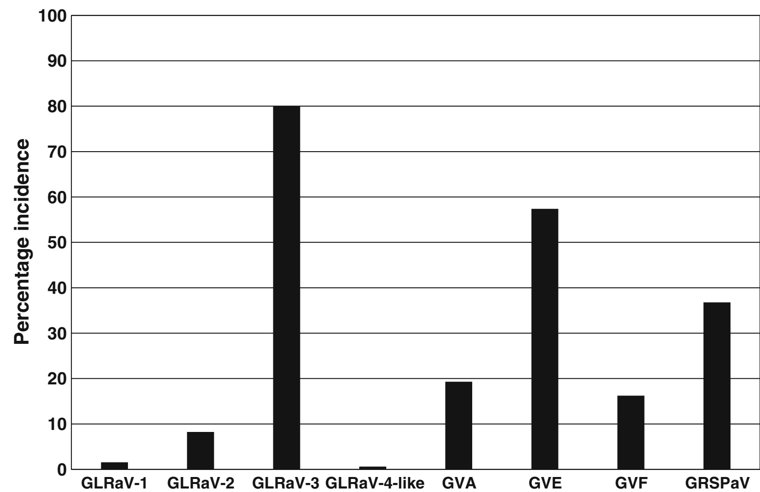


**Fig. 4** Total percentage infection of different viruses detected in vineyards ($\chi^2_{(df=7)}$=648.93 $P$<0.001), indicate significant proportion differences between viruses

**Table 3** Number of positive samples for each virus per cultivar

| | GLRaV-1 | GLRaV-2 | GLRaV-3 | GLRaV-4 | GVA | GVE | GVF | GRSPaV | *N* |
|---|---|---|---|---|---|---|---|---|---|
| Alicante bousche | 1 | 0 | 4 | 0 | 1 | 2 | 1 | 2 | 11 |
| Barbera | 0 | 0 | 3 | 0 | 1 | 0 | 3 | 1 | 8 |
| Cabernet franc | 0 | 1 | 12 | 0 | 3 | 10 | 1 | 0 | 27 |
| Cabernet sauvignon | 1 | 0 | 36 | 0 | 5 | 21 | 3 | 14 | 80 |
| Cape Riesling | 0 | 0 | 4 | 0 | 2 | 1 | 1 | 0 | 8 |
| Carignan | 0 | 1 | 5 | 0 | 1 | 6 | 1 | 3 | 17 |
| Chardonnay | 1 | 1 | 28 | 0 | 11 | 26 | 5 | 10 | 82 |
| Chenin blanc | 0 | 0 | 16 | 0 | 5 | 9 | 2 | 5 | 37 |
| Colombar | 0 | 1 | 8 | 0 | 6 | 0 | 2 | 0 | 17 |
| Granach | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 |
| Malbec | 0 | 1 | 4 | 0 | 0 | 4 | 1 | 1 | 11 |
| Merlot | 0 | 4 | 22 | 1 | 5 | 18 | 3 | 21 | 74 |
| Mourverde | 0 | 0 | 3 | 0 | 0 | 3 | 1 | 2 | 9 |
| Muscat d'Alexandra | 0 | 3 | 1 | 0 | 0 | 2 | 1 | 0 | 7 |
| Muskadel wit | 0 | 0 | 1 | 0 | 0 | 2 | 2 | 2 | 7 |
| Nouvelle | 1 | 3 | 9 | 0 | 1 | 6 | 1 | 6 | 27 |
| Pinot Noir | 0 | 0 | 7 | 0 | 2 | 5 | 3 | 7 | 24 |
| Pinotage | 0 | 0 | 7 | 0 | 6 | 2 | 3 | 1 | 19 |
| Roobernet | 0 | 0 | 4 | 0 | 1 | 2 | 0 | 0 | 7 |
| Ruby Cabernet | 0 | 0 | 2 | 0 | 1 | 0 | 0 | 1 | 4 |
| Sauvignon blanc | 0 | 6 | 22 | 0 | 4 | 17 | 3 | 15 | 67 |
| Semillon | 0 | 1 | 5 | 0 | 0 | 2 | 3 | 2 | 13 |
| Shiraz | 0 | 1 | 35 | 0 | 5 | 27 | 6 | 14 | 88 |
| Viognier | 1 | 1 | 9 | 1 | 1 | 13 | 3 | 8 | 37 |
| Zinfadel | 0 | 2 | 4 | 0 | 0 | 2 | 1 | 0 | 9 |

*N* = Total number of positive samples per cultivar

plants. The variant group II/VI combination was detected as the primary mixed infection in all regions and was well distributed between cultivars. In a previous study, the predominant occurrence of variant group II was shown in 10 mother blocks in South African vineyards (Jooste et al. 2011). Results of this earlier study also showed the spread of variant group II to be faster in a specific disease cluster compared to variant group III (Jooste et al. 2011). This fact may explain the faster spread of variant group II per row in a vineyard. At the time of the earlier study variant group VI had not yet been identified and the spread of this variant was not investigated. The widespread detection of variant groups II and VI in this survey suggest that these two variants are transmitted more effectively to adjacent plants in a disease cluster. A study is underway to test the transmission efficiency of GLRaV-3 variants. Reports from other countries did not show the dominant presence of variant groups II and VI in plants. In China, group I was the most prevalent variant group identified (Farooq et al. 2013), while in the Napa Valley both variant groups I and III were the utmost variants detected (Sharma et al. 2011). In a limited survey in New Zealand, variant groups I and VI were the most frequently detected variant groups (Chooi et al. 2013b). It is interesting to note that variant group III was found in high numbers in the Napa Valley, but in South African vineyards this variant was detected in only eight plants, representing less than 2 % of the tested plants. The overall distribution of isolates similar to the recently described isolate GH24 (GH24-like) was just over 10 %. GH24-like was detected significantly in red and white cultivar vines in the Breede river valley, Cape south coast and Coastal regions.

More GLRaV-3 variants have been identified and recently described. Comparison of different variant groups showed two main phylogenetic clades of the virus (Maree et al. 2013): the clade that include isolates from groups I-V and the clade containing the group VI and group VI-like (NZ2) isolates. A revision of the second phylogenetic clade, group VI and group VI-like, should be done to include the other divergent variants detected, i.e., GH24 and GTG10. In future, full-length coat protein sequences, and preferably full genome sequences, of all newly identified GLRaV-3 variants should be compared to clarify the phylogenetic position of these variants.

The same RNA samples were analysed for the presence of other Clostero-, Viti- and Foveaviruses. Results were obtained for the presence of GVA, GVE, GVF, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-like and GRSPaV. A widespread survey was conducted in this study and GLRaV-3 was found to be the primary virus detected in the plants. A less than 2 % infection rate was observed for other Closteroviruses, namely: GLRaV-1, and GLRaV-4 like viruses. GLRaV-2 was detected in 8 % of plants. Chinese vineyards showed a higher percentage occurrence of other GLRaVs with GLRaV-1, GLRaV-2 and GLRaV-4 occurring at 24.9, 15.3 and 0.8 %, respectively (Liu et al 2013). The Vitivirus, GVE, was detected in significant percentages, 57.5 %, in these plants and GVA and GVF at 19 and 16 % respectively. The Foveavirus, GRSPaV, was the third most prevalent virus in this study detected in 37 % of plants. The detection of the different viruses was based on primers designed in previous studies (for the detection of GVA, GLRaV-1, GLRaV-3, and GRSPaV) and primers designed based on sequence data deposited in GenBank (for the detection of GVE, GVF, GH24-like, GLRaV-2 and GLRaV-4-like viruses). Detection is limited to the accuracy and sensitivity of the primers used and we present the results based on this fact.

Most plants (80 %) tested positive for mixed virus infections and only 9.2 % of plants were singly infected with GLRaV-3. The infection with multiple viruses has a direct effect on the symptom expression of infected plants. Plants showing a typical leafroll symptom might be infected with GLRaV-3 and one to four other viruses leading to the mild or severe leafroll symptoms observed. Mixed virus populations occur more frequently in South African vineyards compared to results from the Napa Valley. There, 81 % of tested plants had single infections of GLRaV-3 (Sharma et al. 2011), a much higher relative abundance of single infected plants when compared to South African vineyards. Recently, results from a Chinese survey showed that single GLRaV-3 infections were found in 66.3 % of plants (Liu et al. 2013) which is in contrast to this study where mixed infections were found to be most common. This is also the case with the GLRaV-3 variants, where 41 % of plants were infected with multiple GLRaV-3 variant groups in South African vineyards opposed to 22 % multiple infected plants in the Napa Valley study (Sharma et al. 2011). In a survey for GLRaV-1, GLRaV-2 and GLRaV-3 conducted in the Finger Lakes vineyards in New York, single infections

occurred in 10, 3 and 15 % of plants, respectively; mixed infections affected only 3.6 % of plants (Fuchs et al. 2009). Although the presence of only three viruses was studied in the Finger Lakes district, the low percentage of mixed infected plants are in contrast to what we observe in South African vineyards. A study from Turkey showed a higher prevalence of GLRaV-1 in their vineyards compared to GLRaV-3 (Akbas et al. 2007). The Turkish study noted that mixed infections are common in vines affected by leafroll-associated viruses and detected mixed infections between different GLRaVs. The survey of GLRaVs from Tunisia showed that 45.8 % of plants tested were infected with more than one virus (Mahfoudhi et al. 2008). GLRaV-3 was the most widespread virus, 76.3 % in Tunisian vineyards, followed by the GLRaV-4-like viruses (GLRaV-5, -6) then GLRaV-1, GLRaV-2, and GLRaV-7, the last three occurring with less than 10 % infection (Mahfoudhi et al. 2008). In Chile, a low percentage infection was reported for GLRaVs and the high relative abundance of GLRaV-2 in their vineyards was reported (Fiore et al. 2008). In China mixed infections with GLRaV-1 and GLRaV-3 were the most common (Liu et al. 2013). It is clear that in certain parts of the world certain viruses occur predominantly. The influence of mealybug vectors in the transmission efficiency of GLRaV-3 variants and other virus populations needs to be studied.

This study demonstrated the complexity of virus- and GLRaV-3 variant infections in South African vineyards. Despite the fact that plants displaying GLD symptoms were targeted for the survey, 20 % (mainly white cultivars) of the collected plants were not infected with GLRaV-3. Correct identification of virus infection based on visual inspection of vineyards is therefore not possible in certain white cultivar plants, which do not display distinct symptoms. This highlights the necessity to have accurate and sensitive detection methods in place to screen plants, especially when screening planting material that will be distributed by industry to producers.

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