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FINAL REPORT
2014

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Project Information

Research Organisation Project number	GenUS 11-01
Project title	Survey of white and red cultivar vineyards affected by grapevine leafroll disease for genetic variation in grapevine leafroll-associated virus 3.

Fruit kind(s)	Grapevine		
Start date (mm/yyyy)	01/01/2012	**End date** (mm/yyyy)	31/12/2013

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

Executive Summary

A survey of viruses affecting grapevine in the wine regions of the Western Cape Province in South Africa was conducted. The survey determined the incidence 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 prevalent as single infections and in combinations. The distribution of variants per region was analysed and it was found that single infections of variant groups II and VI were distributed predominantly in certain regions and were equally distributed in the red and white cultivars studied. The distribution of a recently identified GLRaV-3 (unclassified variant) was also included in the study. The overall analysis showed that variant groups II and group VI were distributed equally among the samples (38% and 36% infection, respectively), followed by variant group I with 12.5% infection and variants similar to isolate GH24 and variant group III with 10% and 2% infection, respectively. GLRaV-3 was the predominant virus detected in the samples and contributed towards 40% of the total viral composition, followed by GVE (24%), GRSPaV (15%), GVA (8%), GVF (6%), GLRaV-2 (3.5%) and GLRaV-1 (0.7% infection). Most of the tested plants were infected with multiple viruses. Mixed virus infections were found in 270 plants representing 34 virus combinations. The foremost virus combination was GLRaV-3 with GVE found in 28% of the plants. 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 GLD.

Problem identification and objectives

The *Ampelovirus*, grapevine leafroll associated virus 3 (GLRaV-3), is the most widespread virus in South African vineyards. The molecular variability of the virus was studied in more detail during the past five years from different regions, especially in South Africa, Portugal and the USA (Fuchs et al., 2009, Jooste et al., 2010, Gouveia et al., 2010, Sharma et al., 2011, Bester et al., 2012a). The availability of more full-length sequences is significantly accelerating the genetic study of GLRaV-3 variants.

To date, six, or possibly seven genetic variant groups of GLRaV-3 were identified world-wide (Maree et al, 2008., Jooste et al., 2010, Gouveia et al., 2010., Sharma et al., 2011, Bester et al, 2012, Chooi et al.,2013). Six full genome sequences, representing four of the genetic variant groups of GLRaV-3, were 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), group VI (represented by isolates GH11, GH30) (Maree et al., 2008, Jooste et al., 2010., Bester et al., 2012).

In previous studies GLRaV-3 variants were identified based on single-strand conformation polymorphism (SSCP) of a genomic region in ORF5 (Jooste et al., 2010, 2011). This technique was able to identify variants from groups I, II and III, but was unable to detect variants from group VI.

The aims of the project:

- In this study an improved detection method, a one-step real-time reverse transcriptase polymerase chain reaction (qRT-PCR) followed by high-resolution melting (HRM) curve analysis, was optimized and used in two surveys (2012/2013) of field collected plants in the Western Cape Province. A detailed description of this technique was published (Bester et al., 2012b).
- The variant status of field collected samples from two surveys was determined.
- The prevalence of the newly described group VI variants in South African vineyards was studied, and possibility of additional GLRaV-3 variants evaluated.
- Additionally, the virus profiles were determined for individual vines.

Workplan (materials and methods)

Survey and sampling

A widespread survey was conducted in the wine growing regions of the Western Cape Province, South Africa. The samples were collected from 3 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 main cultivars (Chardonnay, Sauvignon blanc, Cabernet Sauvignon, Merlot, Shiraz) grown in South Africa. The vineyards selected for the study were all previously used as propagation material for the grapevine industry but lost their status in the 2008/2009 (for plants selected for the 2012 survey) and 2010/2011 (for plants selected for the 2013 survey) growing seasons due to leafroll infection of more than 3%, based on symptom expression. These vineyards were selected to collect data on newly infected plants assuming that the infection was transmitted from surrounding plants. Petioles and canes from four infected plants were randomly sampled in vineyards. In red cultivars leafroll symptoms were easily visible and sampling were done to include a range of leafroll symptoms, from severely dark red and downward curling plants to the plants with milder leafroll symptoms. In white cultivars, leafroll symptoms were not always prominent on the plants, except for Chardonnay plants where clear downward leafcurl was observed. In white cultivar blocks sampling were 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^{o}C and total RNA extracted from 0.2 g tissue using a modified CTAB method (2% CTAB, 2.5% PVP-40, 100 mM Tris-HCL pH8, 2M NaCl, 25 mM EDTA pH8 and 3% β-mercaptoethanol) (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 RT-PCR high-resolution melting curve (HRM) assay was used (Bester et al, 2012b). 100 ng-200 ng of purified total RNA was used as template in the real-time RT-PCR HRM assay that was performed on a Qiagen

Rotor-Gene Q instrument. A 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. All intervals were used as described previously except the interval for variant group I. More data points were included to refine this confidence interval. Samples identified in the 2013 survey as being potentially positive for only variant group I were flagged and screened with the multiplex RT-PCR described in Bester et al. (2012b) to confirm variant group I status. After confirmation, all variant group I melting curve data points of this study were added to the existing melting curve data points (Bester et al., 2012b) to re-calculate the new confidence intervals for variant group I for both LR3.HRM4 and LR3.HRM6 primer pairs.

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 (Table 1). Positive controls for *Grapevine virus A* (GVA), *Grapevine virus F* (GVF), *Grapevine leafroll-associated virus 1* (GVRaV-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. 200 mg of each sample was pulverised with mortar and pestle using liquid nitrogen, and total RNA extracted following an adapted CTAB method (White et al., 2008).

Table 1. List of primers used in RT-PCR and real time RT-PCR HRM assays*. GenBank accessions used for primer design in this study are listed.

Target Virus	Primer Pairs	Sequence (5'- 3')	Amplicon size	Reference/GenBank Accession Numbers
	GVA-P-F-7038	AGG TCCACGTTTGCTAAG		
GVA	GVA-P-R-7273	CATCGTCTGAGGTTTCTACTA	238	MacKenzie, 1997
	GVE_1_Diag_1_7055F	ATGATTTGATGCTCAGTCACAGG		This study (GU903012.1; JX402759.1; AB432910.1;
GVE	GVE_1_Diag_1_7251R	GGGTTCTTATGGCCTGCTTA	213	NC_011106.1)
	GVF_PRIMER_F	TTGGGAGTGGAGGATCTGTA		
GVF	GVF_PRIMER_R	ATGGGCAGTCTGGGTCTATC	217	This study (JX105428.1; NC_018458.1)
	LR1_HSP70-417F	GAGCGACTTGCGACTTATCGA		
GLRaV-1	LR1_HSP70-737R	GGTAAACGGGTGTTCTTCAATTCT	320	Osman et al., 2007
	LR2-P24-F	ATGAGCTCATGAGGGTTATAGTGTCTC		This study (JX559644.1; NC_007448.1; DQ286725.2;
GLRaV-2	LR2-P24-R	ATTCTAGATTAACATTCGTCTTGGAGTTCG	617	AY881628.1)
	GLRaV-3 ORF10 For	AGAGCTCATGGACCTATCGTTTATTAT		??
GLRaV-3	GLRaV-3 ORF10 Rev	ATCTAGAGGCTTGACAACACAACATT	561	
	LR3.HRM4.F	TAATCGGAGGTTTAGGTTCC		
GLRaV-3*	LR3.HRM4.R	GTCGGTTCGTTAACAACAC	226	Bester et al., 2012b
	LR3.HRM6.F	GTCACCAGGTGTTCCAAACC		
GLRaV-3*	LR3.HRM6.R	AACGCCCTGTATGTCCTCTC	305	Bester et al., 2012b
	CB19_72F	GCGAAGACGGATACTGTATCGATA		
GH24-like	CB19_1267R	CACGACCCCTATATCAGCCG	1195	Unpublished
	GLRaV-4.like.F	ATGGCATTGTCTGCGACTAG		
GLRaV-4-like	GLRaV-4.like.R	TAAACACAGACATGGGAGTAGC	354	This study (NC_016416.1; NC_016081.1; AY297819.1)
	RSP13-F	GATGAGGTCCAGTTGTTTCC		
GRSPaV	RSP14-R	ATCCAAAGGACCTTTTGACC	338	Meng et al., 1999

Complimentary 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 minutes at 65ºC before placing on ice for 2 minutes to complete the primer annealing reaction. A solution containing 5x AMV RT Buffer (Thermo Scientific), 10 U AMV reverse transcriptase (Thermo Scientific), 10 mM dNTPs (Thermo Scientific) and dH_2O was then added to the 5 µl primer annealing reaction and incubated for 60 minutes at 48ºC to produce cDNA.

A PCR reaction containing 10x KAPA Taq buffer A (KAPA Biosystems), 10mM dNTP's (Thermo Scientific), 20 mM forward primer (Integrated DNA Technologies), 20 mM reverse primer (Integrated DNA Technologies), 2 µl 10x cresol, 5 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 (Table 2); all included an initial denaturation step at 94°C for 3 minutes and a final extension step at 72°C for 7 minutes.

Table 2. PCR cycling conditions for detection of other Clostero-, Viti- and Fovea viruses

Primer Pair	Initial Denaturation		Denaturation		Annealing		Extension		Cycles	Final Extension	
	Temp (°C)	Duration (sec)	Temp (°C)	Duration (sec)	Temp (°C)	Duration (sec)	Temp (°C)	Duration (sec)		Temp (°C)	Duration (sec)
GtA					56						
GtE					60				45		
GtF					56						
GtRuv1					54/56				30 – 45		
GtRuv2	94	180	94	30	58/56	30	72	60	30 – 35	72	420
GtRuv3					58				35		
GtRuv4 like					62/60				30 – 35		
GtG10					60				45		
GtSPav					53						

Statistical analysis of data

The categorical data was summarised in one-way and two-way classifications. For the one-way classifications a χ^2one-sample test was performed to test for significant differences between the proportions. For the two-way classifications a test for a RxC table was performed to test for similar patterns or independence (Sedecor and Cochran, 1980; Siegel, 1956). A χ^2test 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, 1999).

Results and discussion

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, GTG10, similar to isolate GH24. Positive isolates hereafter referred to as GH24-like.

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. 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℃ and for LR3.HRM6 as 84.82℃ to 85.90℃ (Table 3).

Table 3: Descriptive statistics and re-calculation of variant group I HRM confidence intervals

Primer pair	Number of data points		Min		Max		Mean		Temperature range between upper and lower limit	
	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted
LR3.HRM4	31	60	83.20	83.20	83.98	84.10	83.60	83.68	0.78	0.90
LR3.HRM6	27	63	84.78	84.78	85.42	86.08	85.03	85.33	0.64	1.30

	2.5th percentile		97.5th percentile		Interquartile range (IQR) (75%-25%)	
	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted	Bester et al., 2012	Adjusted
LR3.HRM4	83.22	**83.22**	84.08	**84.05**	0.43	0.37
LR3.HRM6	84.79	**84.82**	85.39	**85.90**	0.09	0.52

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

119 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 were detected in all the districts (Figure 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 and in Worcester district only one plant with a variant group VI infection was detected. In the Cape South Coast region, the Walkerbay district, variant group VI was the only single variant infection detected in five plants ($\chi^2_{(df=9)}$=113.69 P<0.001) (Figure 1A).

Mixed variant infections

As expected, a high number of plants tested positive for mixed variant infections. Fourteen variant combinations were detected in 130 plants. In total, 56.25% of plants

collected in the Breede river valley region was 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 mixed variant infections were more than 20% higher in the Breede river valley. 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) (Figure 1B).

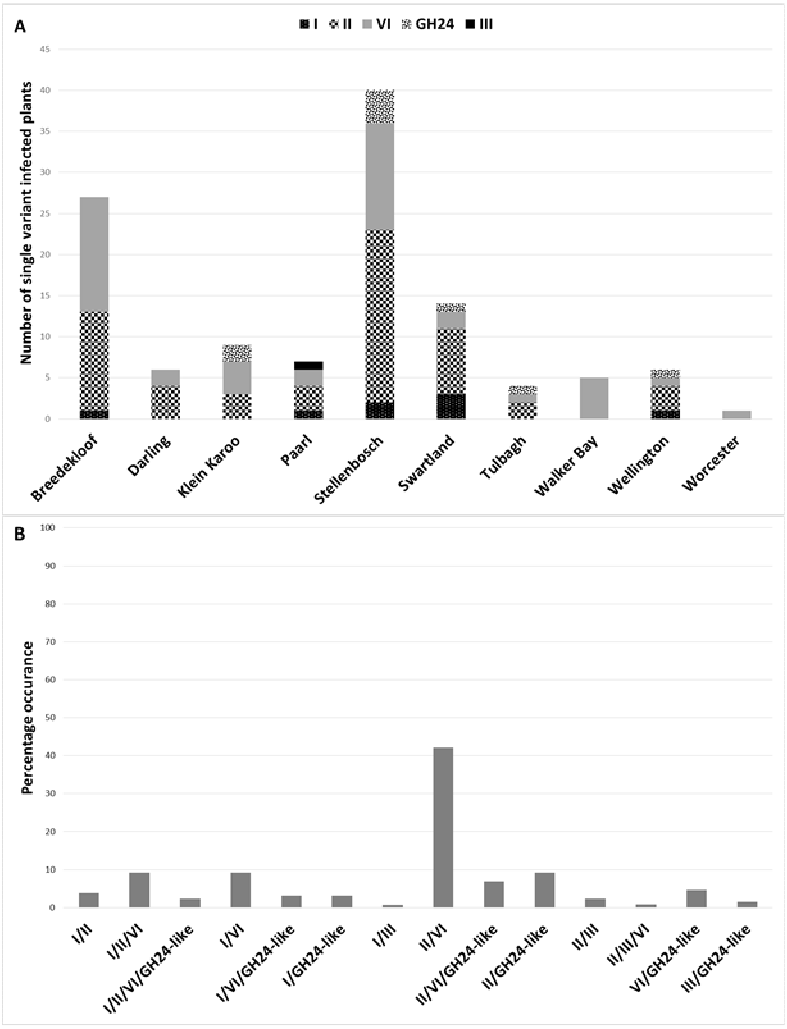

Figure 1. Distribution of single variant infections per district (A) and percentage occurrence of 14 mixed variant combinations in vineyards with variant groups II/VI dominating (B).

The total distribution of GLRaV-3 variants in red and white cultivars were analysed in the three regions (Figure 2A). Variant of group VI occurred dominantly in the Walker Bay district in the Cape south coast region (Figure 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 P=0.847) (Figure 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. Although the chi-square test was not significant, variant group III was only detected in the Coastal region. The overall distribution of variants are seen in Figure 2C.

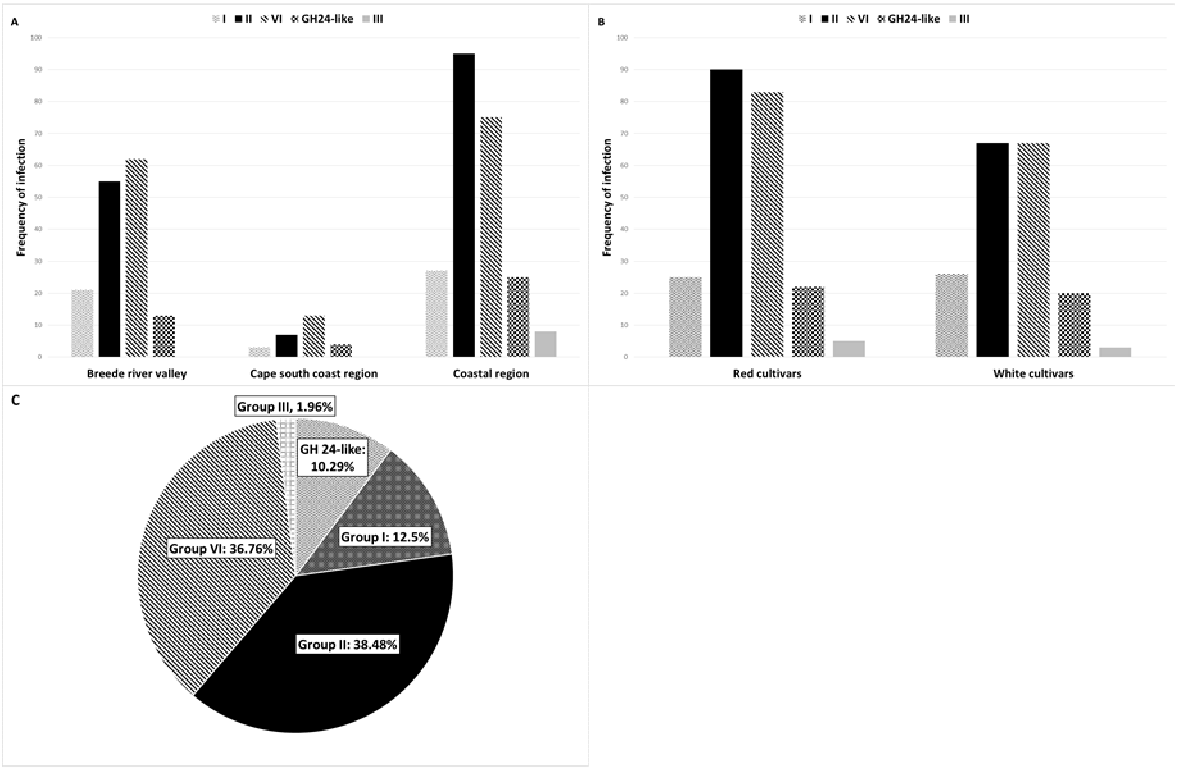

Figure 2. The total GLRaV-3 variant distribution per region (A); the total GLRaV-3 distribution in white and red cultivars (B) and the overall GLRaV-3 variant percentage infection detected in vineyards (C).

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 reactions to detect GVA, GVE, GVF, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4–like viruses and GRSPaV. There were 39 plants with single virus infections of GLRaV-3 and GRSPaV. GLRaV-3 was a single infection in 38 of these plants and GRSPaV was found as a single infection in one plant ($\chi^2_{(df=1)}$=35.1 P<0.001).

Most of the plants tested were infected with multiple viruses. Mixed virus infections were found in 270 plants representing 34 virus combinations ($\chi^2_{(df=33)}$=868.62 P<0.001), indicating that proportions are significantly different. A summary of the mixed virus combinations can be seen in Table 4. The four most prevalent mixed virus infections detected, between 7% and 27%, were: GLRaV-3/GVE, GLRaV-3/GVE/GRSPaV, GLRaV-3/GRSPaV and GLRaV-3/GVA.

Table 4. Mixed virus infections detected in 270 plants of the survey. The four main virus combinations are highlighted.

Mixed virus combination	Number of plants infected	% Infection
GLRaV-1/GLRaV-3/GRSPaV	2	0.74
GLRaV-2/GLRaV-3	4	1.48
GLRaV-2/GLRaV-3/GRSPaV	1	0.37
GLRaV-3/GLRaV-4	1	0.37
GLRaV-3/GRSPaV	32	11.85
GVA/GLRaV-3	21	7.8
GVA/GLRaV-3/GLRaV-4	1	0.37
GVA/GLRaV-3/GRSPaV	5	1.85
GVA/GVE/GLRaV-3	14	5.2
GVA/GVE/GLRaV-3/GRSPaV	8	3
GVA/GVE/GVF/GLRaV-2/GLRaV-3	1	0.37
GVA/GVE/GVF/GLRaV-2/GLRaV-3/GRSPaV	1	0.37
GVA/GVE/GVF/GLRaV-3	6	2.2
GVA/GVE/GRSPaV	1	0.37
GVA/GVF/GLRaV-3	2	0.74
GVA/GVF/GLRaV-3/GRSPaV	1	0.37
GVE/GLRaV-1/GLRaV-3	2	0.74
GVE/GLRaV-2/GLRaV-3	7	2.6
GVE/GLRaV-2/GLRaV-3/GRSPaV	5	1.85
GVE/GLRaV-2/GRSPaV	2	0.74
GVE/GLRaV-3	75	27.8
GVE/GLRaV-3/GRSPaV	35	13
GVE/GVF/GLRaV-1/GLRaV-3	1	0.37
GVE/GVF/GLRaV-2/GLRaV-3	2	0.74
GVE/GVF/GLRaV-2/GRSPaV	1	0.37
GVE/GVF/GLRaV-3	9	3.3
GVE/GVF/GLRaV-3/GRSPaV	6	2.2
GVE/GVF/GRSPaV	1	0.37
GVE/GRSPaV	2	0.74
GVF/GLRaV-2/GLRaV-3	1	0.37
GVF/GLRaV-2/GLRaV-3/GRSPaV	1	0.37
GVF/GLRaV-3	9	3.3
GVF/GLRaV-3/GRSPaV	9	3.3
GVF/GRSPaV	1	0.37

The distribution of these viruses per region is shown in (Figure 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 14% and 20% 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.

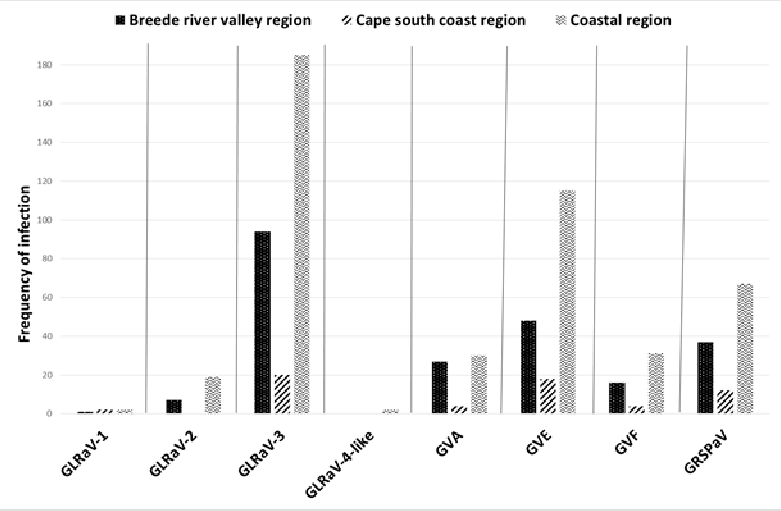

Figure 3. Distribution of viruses in three regions of the Western Cape: Breede river valley region ($\chi^2_{(df=7)}$=344.16 P<0.001), Cape south coast region ($\chi^2_{(df=7)}$=91.84 P<0.001), Coastal region ($\chi^2_{(df=7)}$=713.16 P<0.001)

The total distribution of viruses is summarised in Figure 4 ($\chi^2_{(df=7)}$=795.12 P<0.001).

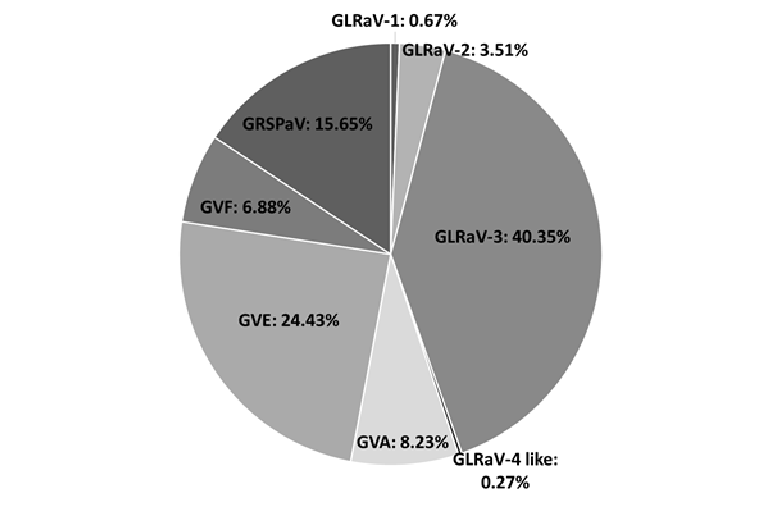

Figure 4. Total percentage infection of different viruses detected in vineyards ($\chi^2_{(df=7)}$=795.12 P<0.001)

Benefits of study to industry

1. Virus profiles were determined for the surveyed plants including GLRaV-3 variant status of plants and infection with other Clostero- Viti- and Fovea viruses
2. With this knowledge suitable detection systems can be put in place in order to detect all known viruses in South African vineyards
3. This study demonstrated the complexity of virus infections in South African vineyards. The fact that white cultivar plants also contain a lot of viruses without symptom expression support the need to be able to test for viruses in white cultivars.

Complete the following table

Milestone	Target Date	Extension Date	Date Completed	Achievement
1. Optimised universal detection methods (qRT-PCR HRM, RT-PCR) to known GLRaV-3 variants	October 2012		October 2012	
2. Conducted a widespread survey in 2012/2013 in leafroll-infected vineyards (including white cultivars) of the Western Cape to determine strain and genetic diversity of GLRaV-3	April/May 2012/2013		April/May 2012/2013	
3. Determined the incidence of known genetic variants from groups I, II, III and VI, represented by 621, GP18, PL-20 and GH11 in Western Cape vineyards	April/May 20122013		June 2012/2013	
4. Determined the virus status of plants in the survey. Screen for GLRaV-1, Vitiviruses, RSPaV, GLRaV-1, -2,- group 4-like viruses.	October 2013		October 2013	
5. Evaluate the efficiency of current detection methods for detection of GLRaV-3 variants and strains	October 2012			
6. Publish results	December 2014			

Accumulated outputs

Conclusions

In this study, the distribution of GLRaV-3 variants and other grapevine infecting viruses were 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 visual symptomology, especially in the case of certain white cultivars. With the high incidences of GLRaV-3 in these plants we strongly suggest, and confirm previous reports, that GLRaV-3 is the main causative agent of GLD in South African vineyards. Overall, in comparison to other *Clostero-, Viti-, and Fovea* viruses, the GLRaV-3 represented 40% of the viral load. This confirms the importance of GLRaV-3 in South African vineyards and the importance to be able to detect all variants of the virus.

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 real-time 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 group VI was detected prevalently in the Breede river valley region including Breedekloof and Worcester districts while variant group VI showed higher incidence in the Cape south coast region (Figure 2A). The overall distribution of the different variants showed similar distribution in red and white cultivars as shown in Figure 2B. The study highlighted the importance to be 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 270 of the tested 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., 2012), 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 8 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 in red and white cultivar plants significantly distributed 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. GLRaV-3, GVE, GVA and GRSPaV were detected in a previous deep sequencing analysis of a South African vineyard (Coetzee et al., 2010). 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 10% infection rate was observed for other Clostero viruses, namely: GLRaV-1, GLRaV-2 and GLRaV-4 like viruses. The *Vitivirus*, GVE, was detected in significant percentages in these plants and GVA and GVF at 8% and 6% respectively. The *Foveavirus*, GRSPaV, was the third most prevalent virus in this study. 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, 85.7%, tested positive for mixed virus infections and only 12% of plants were singly infected with GLRaV-3. This may have a direct effect on the symptom expression of infected plants with these multiple viruses. 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 incidence of single infected plants when compared to South African vineyards. 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 were studied in the Finger Lakes district, the low percentage of mixed infected plants are contradicting 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 and Ilhan, 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 incidence of GLRaV-2 in their vineyards was reported (Fiore et al., 2008). 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 confirms the importance of being able to detect all possible variants of viruses in vineyards, especially when screening planting material distributed by industry to producers.

This study demonstrated the complexity of virus infections in South African vineyards and contributed to the knowledge of the detection of viruses and variants of viruses.

Literature

Akbas, B., Kunter, B., Ihan, D. 2007. Occurrence and distribution of *Grapevine leafroll-associated viruses* 1, 2, 3 and 7 in Turkey. J. Phytopathol. 155:122-124.

Bester,R.,Maree,H.J., and Burger,J.T.(2012a).Complete nucleotide sequence of a new strain of grapevine leafroll-associated virus3 in South Africa. *Arch.Virol.* 157, 1815–1819.

Bester, R., Jooste, A.E.C., Maree, H.J., Burger, J.T., 2012b. Real-time RT-PCR high-resolution melting curve analysis and multiplex RT-PCR to detect and differentiate grapevine leafroll-associated virus 3 variant groups I, II, III and VI. Virol. J. 9. doi:10.1186/1743-422X-9-219

Coetzee,B., Freeborough,M., Maree,H. J., Celton,J., Rees,D.J.,and Burger, J.T. 2010. Deep sequencing analysis of viruses infecting grapevines: virome of a vineyard. *Virology* 400, 157–163.

Chooi,K.M.,Cohen,D.,and Pearson,M.N.2013. .Molecular chacterisation of two divergent variants of *Grapevine leafroll-associated virus 3* in NewZealand. *Arch.Virol* doi:10.1007/s00705-013-1631-9

Farooq, A.B.U., Ma,Y., Wand, Z., Zhou,N.,and Wenxing,X. 2012. Genetic diversity analyses reveal novel recombination events in Grapevine leafroll-associated virus- 3 in China. *VirusRes.* 171, 15–21.

Fuchs, M., Martinson, T.E., Loeb, G.M., and Hoch, H.C. 2009. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. Plant Disease 93:395-401.

Goszczynski, D.E. 2013. Brief report of a new highly divergent variant of *Grapevine leafroll-associated virus 3* (GLRaV-3). Journal of Phytopathology: doi:10.1111/jph.12139

Gouveia, P., Santos, M.T., Eiras-Dias, J.E., and Nolasco, G. 2011. Five phylogenetic groups identified in the coat protein gene of grapevine leafroll-associated virus 3 obtained from Portuguese grapevine varieties. Archives of Virology: 156:413-420.

Jooste, A.E.C., Maree, H.J., Bellstedt, D.U., Goszczynski, D.E., Pietersen, G., and Burger, J.T: 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5'UTR. Archives of Virology 155:1997–2006.

Jooste, A.E.C., Pietersen, G., and Burger, J.T. 2011. Distribution of grapevine leafroll associated virus-3 variants in South African vineyards. European Journal of Plant Pathology 131:371-381.

Mahfoudhi N., Digiaro, M., Dhouibi, M.H., 2008. Incidence and distribution of grapevine leafroll-associated viruses in Tunisian vineyards. J. Phytopathol. 156:556-558.

Maree, H.J., Freeborough, M-J., and Burger, J.T. 2008. Complete nucleotide sequence of a South African isolate of grapevine leafroll-associated virus 3 reveals a 5'UTR of 737 nucleotides. Archives of Virology 153:755–757.

SAS Institute, Inc. 1999, SAS/STAT User's Guide, Version 9, 1st printing, Volume 2. SAS Institute Inc, SAS Campus drive, Cary, North Carolina 27513

Sedecor, G.W., and Cochran, W.G. 1980. Statistical methods (7th Ed.) Iowa State University Press, pp 507.

Siegel, S. 1956. Non-parametric stistics for the behavioural sciences. New York: McGraw-Hill Book Co. Inc.

Sharma, A.M., Wang, J., Duffy, S., Zhang, S., Wong, M.K., Rashed, A., Cooper, M.L., Daane, K.M., and Almeida, R.P.P. 2011. Occurrence of Grapevine Leafroll-ssociated virus complex in Napa Valley. PLoS ONE 6(10): e26227.doi:10.1371/journal.pone.0026227.

White, E.J., Venter, M., Hiten, N.F., and Burger, J.T. 2008. Modified Cetyltrimethylammonium bromide method improves robustness and versatility: the benchmark for plant RNA extraction. Biotechnology Journal 3(11):1424-1428.

Technology development, products and patents

A real-time RT-PCR HRM detection method was developed that differentiate between GLRaV-3 variants (2012)

Suggestions for technology transfer

Human resources development/training

Student level (BSc, MSc, PhD, Post doc)	Cost to Project
1. Rachelle Bester, MSc (2012)	n/a
2. Nicholas Molenaar, MSc (2013)	n/a

Publications (popular, press releases, semi-scientific, scientific)

Bester, R., Jooste, A.E.C., Maree, H.J., Burger, J.T., 2012.Real-time RT-PCR high-resolution melting curve analysis and multiplex RT-PCR to detect and differentiate grapevine leafroll-associated virus 3 variant groups I, II, III and VI. Virology Journal. 9(219).

Jooste, AEC, Molenaar, N., Maree, HJ., Bester, R, Morey, L., de Koker, WC., Burger, JT. Distribution of GLRaV-3 variants and other grapevine viruses in South African vineyards. Busy to finalise paper for publication at time of report.

Presentations/papers delivered

Jooste E., Bester R. 2012. A survey in white and red cultivars to determine the spread of GLRaV-3 variants in vineyards. Grapevine Virus Workshop XI, Olive Grove, Infruitec, 29 May 2012

Jooste, A.E.C., Bester, R., Maree, H.J., de Koker, W.C., Burger, J.T. A survey of red and white cultivars to test an improved detection technique for GLRaV-3 variants identified in South African vineyards. Proceedings of the 17th Congress of ICVG, Davis, California, USA, October 7-14 2012, pg122-123

Bester, R., Jooste, A.E.C., Maree, H.J. and Burger, J.T. 2012. Sequencing and detection of a new strain of grapevine leafroll-associated virus 3 in South Africa. 34th South African Society for Enology and Viticulture (SASEV) Congress, Stellenbosch,14-16 November 2012.

R. Bester, A.E.C. Jooste, H.J. Maree, J.T. Burger. Real-time RT-PCR high resolution melting curve analysis and multiplex RT-PCR to detect and differentiate between GLRaV-3 variant groups. Proceedings of the 17th Congress of ICVG, Davis, California, USA, October 7-14 2012, pg 140-141

Jooste, A.E.C., Maree, H.J., Molenaar, N., Bester, R., de Koker, W.C and Burger, J.T. Survey of white and red cultivar vineyards affected by Grapevine leafroll disease for genetic variation in grapevine leafroll-assiciated virus 3.SASEV / WINETECH 35th INTERNATIONAL CONFERENCE, Lord Charles Hotel, Somerset West, South Africa, 13 – 15 November 2013

Total cost summary of the project

TOTAL COST IN REAL TERMS	COST	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
YEAR 1	2012					90000	45000		135000
YEAR 2	2013					110000	55000		156000
YEAR 3									
YEAR 4									
YEAR 5									
TOTAL						200000	100000		**300000**