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Distribution of grapevine leafroll associated virus-3 variants in South African vineyards

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Abstract Three genetic variants of *Grapevine leafroll-associated virus 3* (GLRaV-3) were identified from vineyards of the Western Cape, South Africa. In a previous study, three full genome sequences of isolates representing each of the variant groups were determined. The three variant groups were represented by accessions 621, 623 and PL-20, of variant groups I, II and III respectively. A specific single strand conformation polymorphism (SSCP) profile was assigned to each variant which was used as a quick, reliable detection and differentiation method. In this study we analysed the occurrence of these three GLRaV-3 variants in mother blocks in different cultivars and from different vine growing regions using SSCP. The majority of the plants studied, were infected with the group II variant, similar to isolates 623 and GP18. The distribution of three GLRaV-3 variants within a spatio-temporally recorded cluster of diseased plants was studied by means of SSCP profile analysis of ORF5 amplified PCR products. We showed that different GLRaV-3 variants are transmitted to adjacent plants in an infection cluster. Results showed that, in some leafroll disease clusters, the variant that was present in the original GLRaV-3 infected plant of a cluster was transmitted to adjacent plants in a row and across rows.

Keywords Closteroviridae · Epidemiology · Field survey · GLRaV-3 variants · Transmission

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Introduction

Grapevine leafroll (GLR) disease is one of the most important diseases of grapevines, occurring in all grape-producing countries worldwide, including South Africa (Pietersen 2004). Several phloem-limited filamentous viruses, identified as grapevine leafroll-associated viruses (GLRaVs), have been characterized from leafroll infected grapevines (Fuchs et al. 2009a). These viruses include species from the genera *Closterovirus* (GLRaV-2) and *Ampelovirus* (GLRaV-1, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6 and GLRaV-9) and GLRaV-7, not assigned to a genus yet (Fuchs et al. 2009a).

The disease delays ripening of grapevine berries, decreases the accumulation of sugar and ultimately influences the overall quality of the wines. Leafroll symptoms vary depending on environmental conditions as well as the cultivars concerned. Symptoms are best observed in the period between harvesting and shedding of leaves (late summer and autumn). Leafroll symptoms appear as typical downward rolling of leaves with leaf veins that stay green. In red wine cultivars the areas

between the veins turn red, whereas yellowing of the same leaf areas occur in white wine cultivars (Carstens 2002). GLR disease is transmitted through infected propagation material as well as mealybug and soft scale insect vectors (Petersen and Charles 1997; Belli et al. 1994; Sforza et al. 2003; Cabeleiro and Segura 1997a; Cabeleiro and Segura 1997b; Tsai et al. 2008; Douglas and Krüger 2008).

GLRaV-3 is known to be closely associated with leafroll disease and occurs commonly in South African vineyards (Pietersen 2004; Pietersen 2006). The mealybug *Planococcus ficus* is considered the most important vector of GLRaV-3 in South Africa (Douglas and Krüger 2008). Transmission efficiency studies between *P. ficus* and *P. longispinus* showed that the two mealybug species are both efficient vectors for GLRaV-3 in South African vineyards (Douglas and Krüger 2008).

The first report of natural spread of leafroll disease in South Africa was recorded in 1985 (Engelbrecht and Kasdorf 1985). The natural spread of this disease in a vineyard was proven a few years later when 100 healthy LN33 indicators were planted in a leafroll infected vineyard of cv. Tinta Barocca (Engelbrecht and Kasdorf 1990). The first symptoms on the indicator plants appeared 2-3 years after planting and after 7 years 71% of the plants displayed symptoms. GLRaV-3 was detected in all symptomatic plants.

Recently, several epidemiology studies on leafroll disease have been reported from grapevine growing regions world wide. The spatial distribution of GLRaV-3 was studied in vineyards from Spain (Cabeleiro and Segura 2006; Cabeleiro et al. 2008) and the field spread of GLRaV-3 was monitored in these vineyards since 1991 (Cabeleiro et al. 2008). From this study it was clear that there was a correlation between mealybug incidence and virus spread (Cabeleiro et al. 2008). Scale insects were implied as vectors of GLRaV-3 in the Meaño vineyard where slow, but constant spread of the virus was observed (Cabeleiro et al. 2008). In two vineyards, in Portomarín and Goian, in the same study, the virus inoculum originated from infected plant material resulting in a random distribution of the disease. A study of the spread of leafroll disease in a Napa Valley vineyard in California showed that spread of the disease came from neighbouring blocks, heavily infected with leafroll, and mapping results of the disease showed a spread rate increase of more than 10% per year in this block (Golino et al. 2008). The possible causes for the sudden rapid spread of leafroll in vineyards of California were debated and the authors suggested that something fundamental changed in the vineyards, such as vector epidemiology, grower rootstock preferences and/or new leafroll strains that emerged (Golino et al. 2008). A study of vineyards in the Pacific Northwest (PNW) of the U.S.A. documented the presence of several genetic variants of GLRaV-1, GLRaV-2, *Rupestris stempitting-associated virus* and *Grapevine fanleaf virus* in these vineyards (Rayapati et al. 2009). The identification of variants of the viruses is therefore important to understand the spread of a complex disease like leafroll.

Three genetic variants of grapevine leafroll associated virus 3 (GLRaV-3) were identified in vineyards of the Western Cape, South Africa (Jooste and Goszczynski 2005; Jooste et al. 2010; Maree et al. 2008). The GLRaV-3 variants were identified by single strand conformation polymorphism (SSCP) profiles generated from a region amplified in ORF5. SSCP results and sequence data confirmed the three variant groups and a specific SSCP profile was assigned to each variant group. Results showed that SSCP analysis on the region in ORF5 gives a fast and reliable indication of GLRaV-3 variant status in a plant. In many plants, combinations of variants were detected. The full genome sequence of a representative from each variant group, 621 (GQ352631, group I), 623 (GQ352632, group II) and PL-20 (GQ352633, group III), was determined (Jooste et al. 2010). The most variation between the three variants occurred in their 5′UTR.

The spatial distribution and spatial dynamics (changes in distribution patterns) of GLR disease within mother blocks of the South African Certification Scheme were studied intensively from 2001-2007 (Pietersen 2004; Pietersen 2006). Four common distribution patterns of GLR were observed in this study. The most significant distribution pattern identified in local vineyards was secondary spread within vineyards after establishment (Pietersen 2006). The other described means of GLR spread are primary spread by infected plant material, GLR spread from a preceding vineyard and gradients of GLR infected vines associated with proximal leafroll infected vineyards (Pietersen 2006).

No information is currently available on the spread of specific GLRaV-3 variants in vineyards. The recent data on molecular characteristics of three genetic

variants of GLRaV-3 together with the occurrence of GLR infected plants within mother blocks of the certification scheme lead to the objectives of this study: firstly, to determine which variant occurs predominantly in mother blocks and secondly, to investigate disease clusters and the spread of individual GLRaV-3 variants within such a disease cluster.

Methods

Field survey to determine the occurrence of GLRaV-3 variants in mother blocks

Spatio-temporal distribution patterns of leafroll infected plants for the period 2001 to 2005 were observed from mother blocks from different grapevine production areas, including Stellenbosch-, Paarl, Wellington-, Worcester-, and Somerset West grape production areas (Pietersen unpublished results).

Mother blocks are a category of propagation vineyard within the South African Wine Grape Certification Scheme, and generally refer to the second generation of vines derived from nuclear plants. Nuclear plants are plants from which virus was eliminated through heat therapy and meristem tip culture and tested for the presence of any viruses by indexing on indicator vine plants, for GLRaV-1, -2, and -3 by ELISA and for all leafroll-associated viruses and grapevine virus A by immuno-electron microscopy. These plants are maintained in vector-free gauze houses. Mother blocks represent the second propagation generation planted outside the gauze houses, the first generation being foundation blocks. These vineyards may only be established from a foundation block or approved other mother block planting material. Mother blocks are inspected for leafroll disease annually and infected vines either rogued or marked with paint and the canes pruned before planting material is collected from the unmarked vines. Planting material is no longer collected from mother blocks once leafroll infection levels exceed 3%, whereupon these vineyards loose their mother block status. All mother blocks referred to in these studies were at incidences of leafroll below 3% at the initiation of their being monitored for the spatial spread of leafroll.

The relative position of vines showing leafroll symptoms were recorded and plotted in a XY matrix using the row number and vine position as co-ordinates (Pietersen 2004). Leafroll infected plants were recorded in vineyards on a yearly basis based on symptom expression. In numerous disease foci (also referred to as disease clusters) the infection point or starting point of disease spread appeared to begin from a single plant, from where mealybugs transmitted the disease to adjacent plants in rows and across rows forming clusters of GLR infection. Plants were recorded to be positive for GLR when a symptom was first visible and the assumption that actual infection of a plant may have preceded the symptom expression of GLR in a plant by a constant number of seasons, and therefore that appearance of symptoms with time reflects the timing of infection.

Eighty plants were collected from 9 mother blocks (1, 4, 9, 17, 50, 54, 64, 65, M) in different grapevine growing regions (Table 1). Eight plants were randomly selected per block, except for block 4 where nine plants were collected. The plants were chosen based on the vine position and leafroll distribution data collected previously (Pietersen 2004). Additionally, nine plants (V1-9) were collected randomly from five mother blocks (72, 73, 77, 84, and 108) on the Vergelegen Wine Estate. Grapevine plants displaying leafroll symptoms, spatially distant from other infected plants, as well as plants that were part of a heavily infected disease clusters were selected. Cultivars included Cabernet Sauvignon, Merlot, Shiraz, Petit Verdot, red cultivars, and Palomino, a white cultivar, grafted on different rootstocks (Table 1).

Distribution of GLRaV-3 variants in infected disease clusters

The spread of three GLRaV-3 variants, previously identified from South African vineyards (Jooste et al. 2010), was investigated within three leafroll disease clusters. The disease clusters were studied in a Cabernet Sauvignon block in the Worcester region, Western Cape. The identification of GLRaV-3 variants in leafroll infected plants was described in a previous study (Jooste et al. 2010). In this previous study, SSCP results of plants 16, 17 and 20 showed distinct SSCP profiles that indicated specific variant status of the plants (Jooste et al. 2010). The SSCP profiles were correlated with sequence data. Isolate PL-20 is the representative of variant group III and the full genome sequence of PL-20 is available in GenBank® (accession no. GQ352633). SSCP profiles of plant 16 showed a mixed infection

Table 1 Grapevine plants collected from ten mother blocks from different geographical regions and their GLRaV-3 variant status (*nt*=not determined)

Plant no.	Plant position Block/ Row/Plant	Cultivar×Rootstock	Year planted	Region	Variant group
1.1	1/4/62	Cabernet Sauvignon 1CxRichter99 179	1991	Stellenbosch	II
1.2	1/10/17	Cabernet Sauvignon 1CxRichter99 179	1991	Stellenbosch	I
1.3	1/15/37	Cabernet Sauvignon 1CxRichter99 179	1991	Stellenbosch	I
1.4	1/18/2	Cabernet Sauvignon 1CxRichter99 13A	1991	Stellenbosch	II
1.5	1/18/49	Cabernet Sauvignon 1CxRichter99 13A	1991	Stellenbosch	II
1.6	1/22/40	Cabernet Sauvignon 1CxRichter99 13A	1991	Stellenbosch	I+II
1.7	1/30/63	Cabernet Sauvignon 1CxRichter99 13A	1991	Stellenbosch	II
1.8	1/30/113	Cabernet Sauvignon 1CxRichter99 13A	1991	Stellenbosch	II
4.1	4/1/16	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	II
4.2	4/3/122	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	II
4.3	4/4/125	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	I+II+III
4.4	4/7/70	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	I+II+III
4.5	4/8/18	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	I+II+III
4.6	4/9/5	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	II
4.7	4/6/32	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	II
4.8	4/9/252	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	I+II+III
4..9	4/10/124	Cabernet Sauvignon 1Cx101–14 219A	1997	Somerset West	II
9.1	9/2/75	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/Stellenbosch	II
9.2	9/3/6	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	unknown
9.3	9/3/36	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	I
9.4	9/8/64	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	I+II+III
9.5	9/11/30	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	I
9.6	9/11/99	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	nt
9.7	9/12/85	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	II
9.8	9/13/21	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl/ Stellenbosch	I
17.1	17/2/6	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	II+III
17.2	17/3/44	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	II+III
17.3	17/4/120	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	I
17.4	17/12/34	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	II
17.5	17/15/58	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	II
17.6	17/18/82	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	I+II+III
17.7	17/28/27	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	I+II+III
17.8	17/31/4	Cabernet Sauvignon 163IxRichter99 179	1997	Paarl	I+II+III
50.1	50/12/22	Merlot 12x101–14 28A	1992	Paarl	I+II+III
50.2	50/12/64	Merlot 12x101–14 28A	1992	Paarl	II+III
50.3	50/17/21	Merlot 12x101–14 28A	1992	Paarl	II
50.4	50/23/58	Merlot 12x101–14 28A	1992	Paarl	II
50.5	50/30/9	Merlot 12x101–14 28A	1992	Paarl	II
50.6	50/32/24	Merlot 12x101–14 28A	1992	Paarl	II
50.7	50/33/56	Merlot 12x101–14 28A	1992	Paarl	I
50.8	50/34/62	Merlot 12x101–14 28A	1992	Paarl	II
54.1	54/6/54	Cabernet Sauvignon 1CxRichter99 179	1997	Franshoek/Paarl	I+II
54.2	54/12/45	Cabernet Sauvignon 1CxRichter99 179	1997	Franshoek/Paarl	II

Table 1 (continued)

Plant no.	Plant position Block/ Row/Plant	Cultivar×Rootstock	Year planted	Region	Variant group
54.3	54/14/19	Cabernet Sauvignon 1CxRichter99 179	1997	Franshoek/Paarl	II
54.4	54/16/93	Cabernet Sauvignon 1CxRichter99 179	1997	Franshoek/Paarl	II
54.5	54/23/39	Cabernet Sauvignon 1CxRichter99 179	1997	Franshoek/Paarl	II
54.6	54/52/41	Shiraz 22BxRichter99 13	1998	Franshoek/Paarl	II
54.7	54/65/40	Shiraz 22BxRichter99 30B	1997	Franshoek/Paarl	II
54.8	54/66/4	Shiraz 22BxRichter99 30B	1997	Franshoek/Paarl	II
64.1	64/2/77	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	II
64.2	64/8/82	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	II
64.3	64/9/55	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	II
64.4	64/13/12	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	II
64.5	64/23/11	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	II
64.6	64/37/20	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	nt
64.7	64/41/18	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	I
64.8	64/59/22	Petit Verdot 400Dx101–14 219 F	2000	Stellenbosch/Belville	unknown
65.1	65/16/46	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	nt
65.2	65/20/19	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	I
65.3	65/38/116	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	I+II
65.4	65/39/18	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	I+II
65.5	65/40/10	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	II
65.6	65/44/62	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	I
65.7	65/50/44	Cabernet Sauvignon 341Bx101–14 219 F	2000	Klapmuts/Franshoek	II
M1	38/1/9	Palomino	1980	Worcester/Villiersdorp	II
M2	38/3/4	Palomino	1980	Worcester/Villiersdorp	I+II
M3	38/4/12	Palomino	1980	Worcester/Villiersdorp	I
M4	38/4/55	Palomino	1980	Worcester/Villiersdorp	III
M5	38/16/1	Palomino	1980	Worcester/Villiersdorp	II
M6	38/19/7	Palomino	1980	Worcester/Villiersdorp	II
M7	38/31/23	Palomino	1980	Worcester/Villiersdorp	nt
M8	38/42/25	Palomino	1980	Worcester/Villiersdorp	I+II
V1	72/5/136	Shiraz SA99xRichter 110	1999	Somerset West	II
V2	72/16/84	Shiraz SA99xRichter 110	1999	Somerset West	II
V3	73/27/107	Cabernet Sauvignon 46Cx101–14	1999	Somerset West	II+III
V4	77/2/66	Cabernet Sauvignon 46Cx101–14 219A	2002	Somerset West	II
V5	77/16/32	Cabernet Sauvignon 46Cx101–14 219A	2002	Somerset West	II
V6	72/16/78	Shiraz SA99xRichter 110	1999	Somerset West	I+II+III
V7	84/6/44	Cabernet Sauvignon 46Cx101–14 219A	2002	Somerset West	I
V8	84/27/11	Cabernet Sauvignon 46Cx101–14 219A	2002	Somerset West	I+II+III
V9	108/37/19	Cabernet Sauvignon	1999	Somerset West	II

with group II (623) and group III (PL-20) variants, the dominant variant in plant 17 was the group II (623) variant and plant 20 (PL-20) was infected with the group III variant. The three plants all represented the initial infected plant of any given foci of leafroll infection and were recorded to be leafroll infected in 2001 based on symptom expression. The first symptom expression in a plant and the position of the infected

plant was used to reflect the relative time of infection of a plant. We assumed that actual infection of a plant may have preceded the symptom expression of leafroll in a plant by a number of seasons. The history of symptom development of infected plants in the infection cluster, which developed around the initial infected leafroll plants, was recorded in consecutive years. Ten plants directly adjacent to plants 16, 17 and 20 (A-J) were collected in 2008, as seen in Fig. 1, X indicating the originally infected plant. Two adjacent plants on each side of the original plant, in the row, were collected (A–D) and three plants on either side of the infected plant across rows were collected (E–G and H–J).

RT-PCR and SSCP analysis

Isolation of dsRNA, SSCP analysis and cloning were carried out as described earlier (Goszczynski and Jooste 2002). Double-stranded RNA was isolated from the plants in both experiments and SSCP analysis was performed on PCR amplified products from a region in ORF5 with primer sets H420 and C629 as described in Jooste et al. (2010). PCR products of the expected size were purified directly from low melting agarose gels using a Wizard PCR Prep DNA Purification System (Promega). SSCP profiles were generated in 15% polyacrylamide gels, run for two hours at 200 V and stained with ethidium bromide.

Results

Incidence of GLRaV-3 variants in mother blocks

GLRaV-3 specific RT-PCR amplicons of 209nt from ORF5 were successfully obtained from all 80 plants. SSCP profiles were generated, analysed and each unique profile, representing a specific variant group, reported as a percentage of the total number of SSCP profiles analysed. SSCP results of blocks 1, 4, 9, 38, 54, and 64 are shown in Fig. 2. SSCP profiles representing pure sources of the group I variant can be seen in plants 1.2, 1.3, 9.3, 9.5, 9.8, 64.7 and M3. Similarly, the profile for pure sources of the group II variant can be seen in profiles of plants 1.1, 1.4, 1.5, 1.7, 1.8, 9.1, 9.7, 54.2, 54.3, 54.4, 54.5, 54.6, 54.7, 54.8, 64.1, 64.2, 64.3, 64.4, 64.5, M1, M5, M6, 4.1, 4.2, 4.6, 4.7, and 4.9. Profiles of plants infected with combinations of variants can be seen in plants 1.6, 54.1, infected with variant group 1 and II, and plants in block 4, 4.3, 4.4, 4.5 and 4.8, showing more 'complex' profiles suggesting that they represent a mixed infection of group I, II and III variants. Results showing the respective variant groups detected in every plant are summarized in Table 1. The group II variant was detected in 54% of the plants followed by the group I variant infecting 16.2% of the plants studied. The group III variant was detected in only one plant in a pure form, representing 1.4%. Six plants (8.1%) were infected with a combination of group I and group II variants, and four plants (5.3%) were infected with variants from group II and III simultaneously. Eleven plants or 14.9% of all plants were infected with a combination of all three variants. Two plants had unknown SSCP profiles (9.2 and 64.8) and in four plants it was not possible to generate SSCP profiles because of lower concentration of the amplified product.

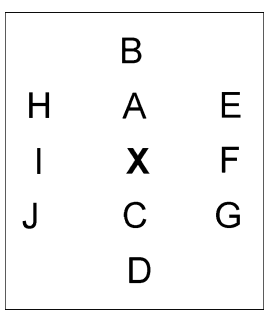

Fig. 1 A diagram of the positions (A-J) of plants collected in a leafroll infected disease cluster relative to the original GLRaV-3 infected plant (X)

Distribution of GLRaV-3 variants in infected disease clusters

Three infection clusters, surrounding plants 16, 17 and 20, were collected and analysed from a vineyard in the Worcester region. The spread of variants from the putative originally infected plant (the first one showing symptoms) to neighbouring plants were studied with SSCP (Fig. 3) in combination with the plotted data of leafroll infected plants from the survey of 2001 to 2005 (Fig. 4). Plant 16, showing leafroll symptoms for the first time in 2001, was infected with variants from group II and III. The plants directly adjacent in the same row, i.e. plants A and B, displayed leafroll symptoms three years later in 2004. The combination of these two variants was transmitted to the two plants along the row. Plants H,

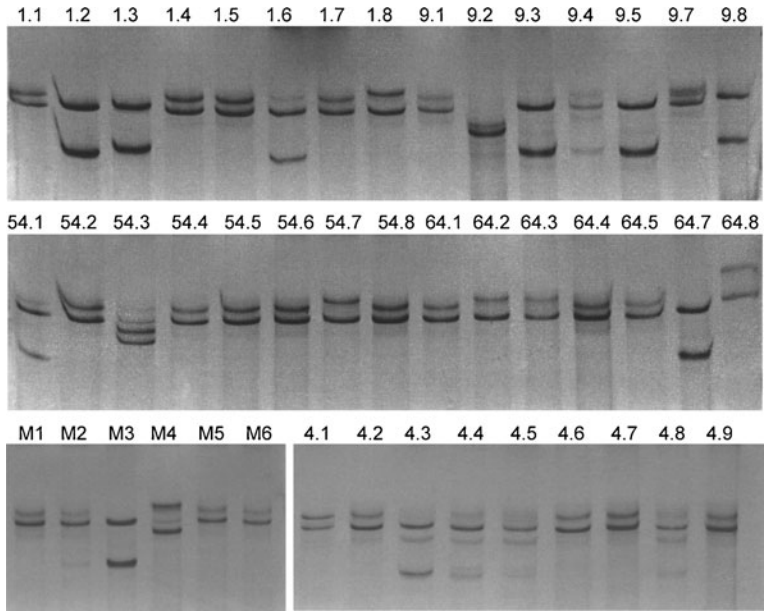

Fig. 2 Examples of SSCP results of ORF5 from plants collected in six mother blocks (1, 9, 54, 64, M and 4). Pure sources of each variant group as well as mixed variant infections were differentiated by unique SSCP profiles

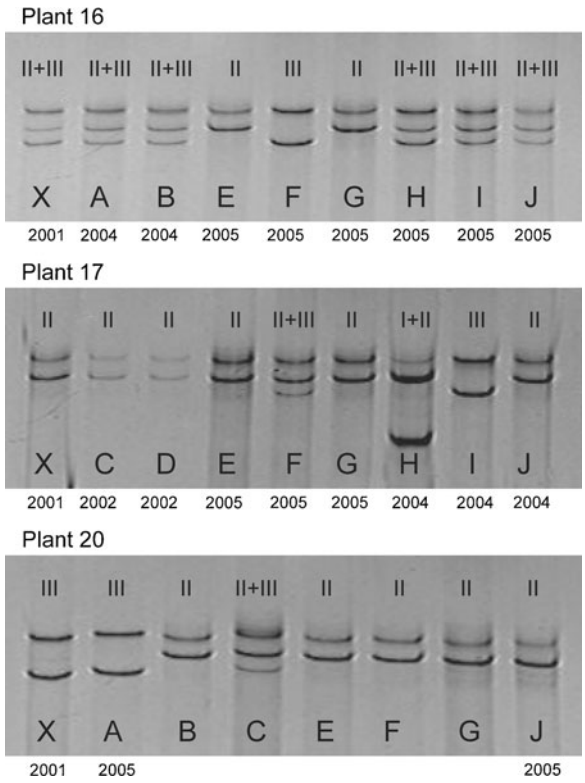

Fig. 3 SSCP analyses of three disease clusters in Block 10 in the Worcester vine growing region, Western Cape. SSCP profiles of eight surrounding plants of plants 16, nine plants surrounding plant 17 and seven plants surrounding plant 20 are shown

I and J, plants across the row, on the right side, showed leafroll symptoms in 2005 and both variants were detected in the plants. Plants E, F and G, in the row on the left of plant 16, showed the separation of group II and III variants. Plants E and G were infected with the group II variant and plant F with the group III variant.

A second infection cluster of plants, those surrounding plant 17, was analysed. Plant 17 was infected with the group II variant and was recorded as leafroll symptomatic in 2001. At that time the adjacent plants did not show any leafroll symptoms. In the following season, 2002, two plants in the row adjacent to plant 17 showed leafroll symptoms and only the group II variant was detected in these plants. Three years after plant 17 was infected with leafroll, in 2004, the plants in the row on the left of the infection foci showed symptoms (plants H, I, and J). Plant H was infected with a combination of variants from group I and II and plants I and J infected with variant group III and II, respectively. Plants E, F, and G, on the left side of plant 17, showed symptoms a year later in 2005 and the group II variant was detected in them as well as the group III variant in plant F.

Plant 20, the representative of the group III variant, displayed symptoms for the first time in 2001 and was an isolated focus point of leafroll disease in the vineyard. Four years later in 2005, plant A, directly next to plant 20 in the row displayed symptoms and was found to be infected with the group III variant.

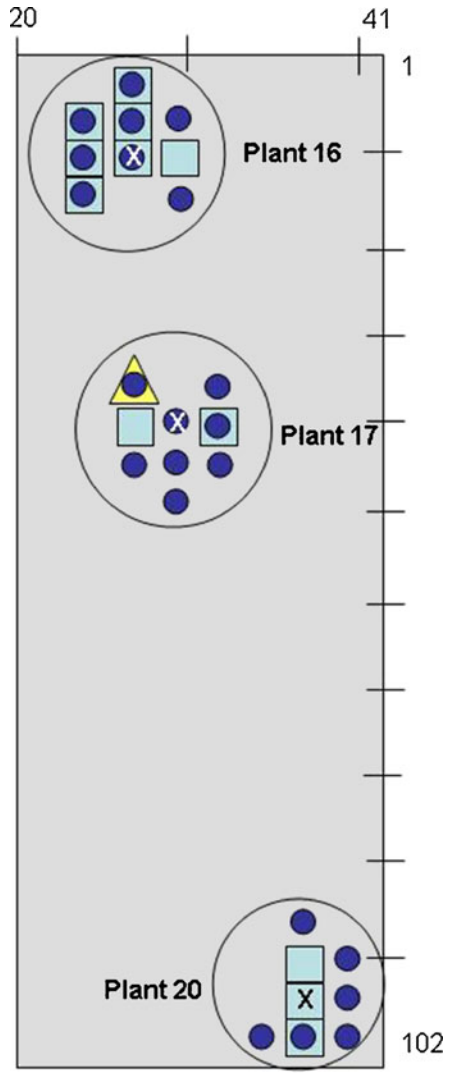

Fig. 4 A graphic presentation of the relative position of the infection clusters in the Worcester vineyard. Rows are indicated horizontally and plant number vertically. The original infected plant of an infection cluster is marked as X, and represents plants 16, 17 and 20, respectively. The GLRaV-3 variant status of every plant in the disease cluster is indicated by the following symbols: variant I (△), variant II (○), variant III (□). Mixed infections with more than one variant is indicated with a combination of the symbols

The other plant recorded to be leafroll infected in 2005 was plant J, diagonally left of plant 20. Plant J was infected with the group II variant. None of the other plants showed symptoms in 2005 and in 2008, when the plants in the cluster were tested, all plants tested positive for GLRaV-3 and contained the group II variant, according to SSCP profiles, except plant C that was infected with a combination of group II and III variants.

Discussion

The identification of three GLRaV-3 variants in local vineyards was investigated, with the focus on the presence of the three GLRaV-3 variants in leafroll disease clusters. A field survey was done; firstly to determine which of the variants occurred predominantly in the selected mother blocks and secondly, to determine if there were any differences in the spatial distribution patterns of three GLRaV-3 variants. This work was linked to the spatial distribution study of leafroll disease within mother blocks of the South African Certification Scheme, investigated from 2001 to 2005 (Pietersen 2004; Pietersen 2006). Distribution data of newly infected leafroll plants within vineyards, based on the visual inspection of diseased plants on a yearly basis, was used as basis for this study. Each of the individual monitored vineyards was planted with the same cultivar, at the same time on the same rootstock and therefore it validates the monitoring strategy based on symptom expression. The distribution of a specific GLRaV-3 variant in a disease cluster was based on the appearance of GLR symptoms in plants.

Phylogenetic analysis of the Hsp70h and coat protein genes in previous studies showed that the majority of GLRaV-3 isolates, deposited in GenBank, grouped into the variant group I clade, represented by the South African accession 621 and American isolate NY-1 (Ling et al. 2004; Fuchs et al. 2009b; Jooste et al. 2010). However, in this study, we determined that variant group II is the predominant variant in the plants collected from 14 mother blocks suggesting that variant II is the most widespread in local vineyards. Whether the predominant occurrence of group II variants reflects a unique situation in vineyards in South Africa is still unknown and warrants further investigation. Many factors, including specific virus-vector interactions, weather conditions, or occurrence of specific GLRaV-3 variants, may contribute to the fact that the group II variant occur predominantly in South Africa. The predominant dissemination of plants with group II variants could also have resulted from the accidental use of infected plant material or infected rootstocks, a known mode of spread (Pietersen 2006). The interaction between a specific GLRaV-3 variant and the ability of the vine mealybug, *P. ficus*, to transmit a specific variant from plant to plant need further investigation.

We also made the assumption that the dominant variant in a plant would be detected with SSCP. The

SSCP profiles characteristic for each variant group, as determined previously (Jooste et al. 2010), correlated with sequence data. Furthermore we assumed that plants with the same SSCP profile shared the same sequence, as observed in the Turturo et al. (2005) study. The upper band of the SSCP profiles in plants 1.8, 54.7, 64.2 and 64.3 (Fig. 3) are slightly higher than the typical SSCP profile (plants 1.4, 1.5, 1.7) for group II variants. Sequence data previously obtained from plants 5 and 48 described in the Fig. 1 in Jooste et al. (2010) showed that a two nucleotide change caused the SSCP profile with the slightly higher upper band. Similarly, the four band profile detected in plant 54.3 was also found in the Jooste et al. (2010) study in plants 7, 12, and 17, and was shown to be group II members based on sequence results. Sequence results obtained from four clones from each plant with a 'duplicate' four band profile proved to be identical to that of plant 5, which has a two band profile, and represent the group II profile (Jooste et al. 2010). Therefore plant 54.3 was identified as a group II variant. Based on SSCP results, the variant status of originally infected plants, plants 16, 17 and 20 in this study, remained the same in two consecutive years, 2007–2008. The mealybug population in these disease clusters were not monitored during the study.

In plants 17 and 20 (Fig. 3), single variant infection were detected, but adjacent plants had mixed variant infections. The single variant infection detected with SSCP in plant 20 (group III variant) only spread to an adjacent plant four years later, suggesting that this variant maybe slower in expressing symptoms or is spread slower by mealybugs in contrast with the group II variant, plant 17, detected in adjacent plants a year later. Plants 20 B, C, E, F and G all tested positive for GLRaV-3 in 2008. The exact year when symptoms became visible were not recorded in these plants. A combination of variants in a plant may be transmitted more effectively to adjacent plants as seen in the transmission of variants in the plant 16 disease cluster. It was not possible to analyse plants in positions 16 C, D, 17 A, B or plants 10 I, H as these positions did not contain vines in the field.

It is likely that plants with older leafroll symptoms are infected with combinations of variants as seen in certain of the SSCP profiles of Fig. 2, for example plants in blocks 4 and 17 (Table 1). This suggests that with time, plants might get infected with a selection of variants, depending on the mealybug's transmission efficiency of different variants. The influence of an insect vector on the change of a viral population was discussed in work done on a well studied *Closterovirus*, *Citrus tristeza virus* (CTV) (Ayllón et al. 1999; d'Urso et al. 2000). SSCP analysis of genes p18 and p20 showed that the profiles characteristic of field CTV isolates were frequently altered after aphid transmission (d'Urso et al. 2000). Similarly, it has been shown (Brlansky et al. 2003) that frequencies of genomic variants in a *Citrus tristeza virus* (CTV) populations may alter following aphid-mediated virus transmission to a new host. Another study by Roy and Brlansky (2009), proved the generation of virus recombinants after aphid transmission. Different dominant genotypes were detected in the parent and aphid-transmitted (AT) sub isolates and even intermediate genotypes were detected that differed from the parental or AT sub isolates (Roy and Brlansky 2009). A study by Broadbent et al. (1996) showed that the influence of aphid transmission sometimes even alters pathogenic characteristics in CTV. Whether mealybug vectors influences the transmission efficiency of specific GLRaV-3 variants or are influencing the viral population in a certain way, needs to be studied.

The classification of GLRaV-3 variants reported in this and previous papers (Jooste and Goszczynski 2005; Jooste et al. 2010) are similar to the phylogenetic classification described by Fuchs et al. (2009a). The Fuchs study classified GLRaV-3 accessions into groups NY-1, GP-18, C5-1, MT48-2 and NZ-1. According to Jooste and Goszczynski (2005) and Jooste et al. (2010) the identified variant groups are numbered I, II and III. The clades identified are similar to the Fuchs study. In the Jooste et al. (2010) paper the genome of isolate 621 (group I) was completely sequenced, and could replace NY-1 as representative of group I. GP-18 is the representative of group II variants and 623 joins this variant group (both complete genomes). PL-20 (complete genome), similar to MT48-2 (partial sequence), from the Jooste et al. (2010) study is the representative of variant group III. We propose that isolates from the NZ-1 clade belong to variant group IV and isolates from the C5-1 clade to variant group V, with representative isolate sequences to be determined when more data is available. In our opinion the proposed Roman numeral classification system for the different variant groups of GLRaV-3 is a sensible approach that will reduce the risk for confusion.

This is the first study to show that a specific GLRaV-3 variant, or a combination of GLRaV-3 variants, is transmitted to adjacent plants in a leafroll infected cluster. As discussed above, the importance of the interaction between the mealybug vector and a specific GLRaV-3 variant warrants further investigations. Mealybug populations in the vineyards and their role in transmitting the GLRaV-3 variants from plant to plant were not monitored in this study. Results of this study concluded that GLRaV-3 variants from group II occur predominantly in the vineyards studied. It is important to continue to study the biological properties of GLRaV-3 variants, including their possible role in causing mild or severe symptoms in grapevine plants.

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