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Accumulated data and results from the recent study of dsRNA isolated from grapevines used in experiments of insect and graft transmission of 'Shiraz' disease

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Summary

RT-PCR amplicons of dsRNA isolated from various grapevines, which were used in the experiments of transmission of 'Shiraz' disease (SD) from 'Cinsaut Blanc' clone P163/12 to SD-susceptible 'Merlot' and 'Shiraz' using mealybug *Planococcus ficus* and grafting were investigated. The amplicons were generated in RT-PCR based on virus-specific or random hexamers oligonucleotide primers. Standard molecular techniques and high-throughput sequencing (HTS), respectively, were applied. The results supported the hypothesis that GVA M5v variant present in 'Cinsaut Blanc' P163/12, which is a member of group II of GVA variants associated with SD, is crucial for developing this disease. HTS data did not reveal any other grapevine viruses besides GLRaV-3 and GVA in SD-affected grapevines, except for GVE which, however, was not present in all diseased plants.

Key words: 'Shiraz' disease; graft and mealybug transmission; GVA variants; RT-PCR; HTS.

Introduction

'Shiraz' disease (SD) is of great concern to the South African grapevine industry since it kills affected grapevines of noble cultivars such as 'Shiraz' and 'Merlot', and is spreading naturally in vineyards. The disease has also been observed on 'Gamay' and 'Viognier'. The main, most characteristic feature of SD is that canes of infected plants do not mature (Fig. 1). Instead, they remain green for an extended period through the season, and are rubbery in texture. Cross-sections of SD-affected canes show underdeveloped xylem and enlarged phloem tissues (Goussard and Bakker 2006). Once grapevines exhibit symptoms of SD the plants never recover. 'Shiraz' disease is latent in grapevines of other cultivars and rootstocks, but it can be transmitted easily from these to SD-susceptible grapevines by grafting with infected tissues. The importance of this highly destructive disease is emphasised by the fact that 'Shiraz' (syn. 'Syrah') cultivar is widely planted in countries like Australia, France, and the USA.

'Shiraz' disease was first described in South Africa in 1985 (Corbet and Wiid 1985). Since then its suspected viral aetiology has remained a mystery. In 2003, we showed that the disease is transmitted by the mealybug, *Planococcus ficus* (Goszczynski and Jooste 2003a), and found that a *Vitivirus*, *Grapevine virus A* (GVA), is associated with the disease (Goszczynski and Jooste 2003a; Goszczynski 2007, Goszczynski and Habili 2012). The study of GVA revealed extensive molecular heterogeneity of this virus in South African vineyards. Three divergent molecular groups of the virus (I, II, III) were identified (Goszczynski and Jooste 2003b). Results showed that the variants of molecular group II are closely associated with 'Shiraz' disease, and variants of molecular group III are present in GVA-infected, SD susceptible grapevines that consistently do not exhibit symptoms of the disease (Goszczynski 2007, Goszczynski and Habili

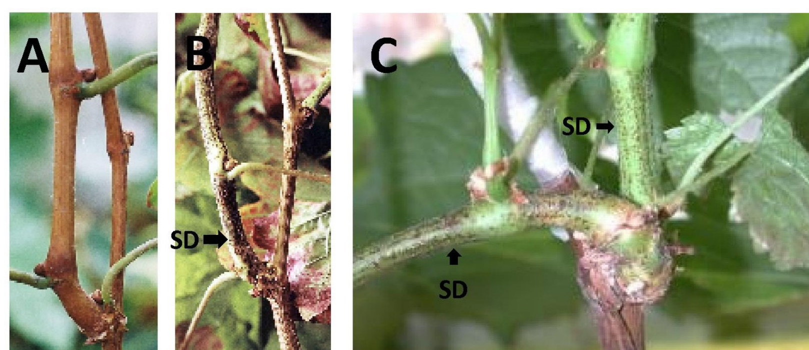

Fig. 1: (**A**) 'Shiraz' disease (SD)-free and (**B**, **C**) SD-affected canes of 'Merlot' in the experiment to transmit this disease from 'Cinsaut Blanc' P163/12 by (**B**) mealybug *P. ficus* and (**C**) grafting.

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2012). A disease that is similar or perhaps identical to SD has also been reported in Australia (Habili 2013).

The 'Cinsaut Blanc' clone 163/12 used in the present study is a unique source of SD. In addition to GVA, this grapevine is clearly infected with *Grapevine leafroll-associated virus* 3 (GLRaV-3) (Goszczynski, unpubl.). The GVA population consists of two genetic variants. The variants were transmitted to an alternative herbaceous host of GVA, *Nicotiana benthamiana*. The full genomes of these variants, named P163-1 and P163-M5 were deposited in GenBank/ EMBL database with accession numbers DQ855088 and DQ855082, respectively. The genomes are highly divergent, sharing only 70.8 % nt similarity (Goszczynski *et al*. 2008). The variant P163-M5 is a member of phylogenetic group II associated with SD in South Africa (Goszczynski and Jooste 2003a, Goszczynski *et al*. 2008). Variants of this group are also commonly present in grapevines affected by 'Shiraz' disease in Australia (Goszczynski and Habili 2012). The variant P163-M5 is unique among variants of group II as it has 119 nt insert in ORF2. The insert sequence shares 75.6 % nt similarity with the corresponding native sequence of this variant, and 68.1-78.2 % nt similarity with other members of the group II, which were identified in South Africa (Goszczynski *et al*. 2008). The biological significance of the modification of ORF2 of GVA-M5 remains unknown. It is important to note that the host of GVA-M5, grapevine 'Cinsaut Blanc' P163/12, was used by grapevine industry as a very reliable source of SD in woody indexing (Maree, Directorate Plant Production, Health and Quality, Stellenbosch, pers. commun.).

Reported here are the results of an analysis of viruses RT-PCR amplified from dsRNA isolated from grapevines used in experiments to transmit SD from 'Cinsaut Blanc' P163/12 to SD-susceptible 'Merlot' and 'Shiraz', using mealybugs and grafting. The experiments were conducted in 2001-2002, and 2011 and 2015, respectively. The aims of this study were as follows: 1. to test the hypothesis of association of GVA-M5 variant with SD, using various newly designed GVA-specific primers; and 2. to investigate whether viruses other than GVA and GLRaV-3 are present in 'Cinsaut Blanc' P163/12, using an HTS analysis of amplicons generated in RT-PCR based on hexanucleotides as primers.

Material and Methods

G r a p e v i n e s: Cane cuttings of 'Cinsaut Blanc' clone P163/12, the source of SD in virus transmissions were received from Directorate Plant Production, Health and Quality, Stellenbosch. Cuttings of the recipient 'Merlot' and 'Shiraz' plant were received from respectively, Ernita nursery in Wellington, and Vititec nursery in Stellenbosch, Western Cape, South Africa. The cuttings were rooted, then potted and kept in a glasshouse. The potted grapevines were propagated from cane cuttings every 5 years. All 'Merlot' and 'Shiraz' plants were consistently free of cane symptoms of 'Shiraz' disease.

T r a n s m i s s i o n o f v i r u s e s u s i n g m e a l y b u g s : A colony of mealybugs, *Planococcus ficus* (Signoret), was established from a single mealybug found on cuttings from naturally infested grapevines collected from a vineyard near Stellenbosch (Western Cape). The colony was maintained on sprouting potatoes at 25 °C in the dark for four months. Then, mealybug-infested potato sprouts were transferred to a plant of 'Cinsaut Blanc' P163/12. The potted plant was kept in a growth room at about 25 °C, with 12 h photoperiod. Mealybugs were allowed to feed on this grapevine for a month, then leaves and fragments of canes that were heavily infested with mealybug crawlers were transferred to 10 *in vitro*-propagated two-month old 'Merlot' plants (received from Ernita nursery). After 2 weeks, mealybug crawlers feeding on 'Merlot' were sprayed with insecticide.

G r a f t i n g o f g r a p e v i n e s: about 10 cm cane cuttings of 'Cinsaut Blanc' P163/12 were manually top grafted to canes of about 3 year-old potted plants of 'Merlot' and 'Shiraz', and kept in growth chambers (about 25 °C, 12 h photoperiod).

I s o l a t i o n o f d s R N A f r o m g r a p e v i n e s: was done as reported by Goszczynski (2007). The procedure is a modification of the method described by Valverde *et al*. (1990). The isolated dsRNA mixed with 2 volume of ethanol was stored in - 30 °C. For reverse transcription (RT), 1 mL of this dsRNA was centrifuged at 19500 rcf for 1 h at 5 °C, and the resulting dsRNA pellet was dried and re-suspended in 10 µL of nuclease-free water. Four µL of it was used in a 10 µ RT reaction.

R T - P C R, c l o n i n g o f a m p l i c o n s, s e q u e n c i n g, S i n g l e S t r a n d C o n f o r m a t i o n P o l y m o r p h i s m s (S S C P) : the techniques were carried out as described by Goszczynski and Habili (2012). Oligonucleotide primers used for amplification of viruses are shown in Tab. 1. Primers V1F, V2R, V3F and V4R were used in RT nested- PCR. Primer pair V1F/ V2R was used in reverse transcription and in the first round of PCR, and V3F/V4R in the second round of PCR (Goszczynski and Habili 2012). The GVA-specific primers MP2F/MP1R, and V1F-V4R were designed in movement protein (ORF3) and in the extensively variable 5' terminal half of replicase (ORF1) genes of the virus, respectively. According to alignments of the primers to all 8 South African GVA variants, which genomes are deposited in GenBank/ EMBL database (Goszczynski *et al*. 2008) (the alignment not shown), the techniques should amplify all the GVA variants (Goszczynski 2007) and only variants of group II (Goszczynski and Habili 2012), respectively. Experiment data revealed however that primers V1F-V4R can amplify, in addition of variants of group II, also variants of group I (Goszczynski and Habili 2012).

H i g h - t h r o u g h p u t s e q u e n c i n g (H T S): dsRNA was RT-PCR amplified using primer P1- 6N (5' GCCGGAGCTCTGCAG AATTCNNNNNN 3'), and the PCR product (clearly visible in agarose gel as smear of about 500-3000 bp DNA sequences) was purified using Wizard SV Gel and PCR Clean-Up System (Promega). HTS technique and the analysis of the obtained sequence data were done at the Biotechnology Plantform, ARC, South Africa. 2 GB data was requested. According to the received report, the raw data were trimmed using Trimmomatics version 0.36, (parameters: Leading:14, Trailing: 14, sliding window: 4:15

Table 1

Virus-specific oligonucleotide primers used in this study

Primer name	Sequence (3' – 5')	Amplicon size (bp)	Specificity	Reference
H7038	AGGTCCACGTTTGCTTAAG	236	GVA	MacKenzie 1997
C7273	CATCGTCTGAGGTTTCTACTA			
H420	GATTTAAGCGCGTTTTTCAGGAC	210	GLRaV-3	MacKenzie 1997
C629	CGGCACGATCGTACTTTCTAA			
MP2F	TCTGAACAAGGCCCTGCA	254	GVA	Goszczynski 2007
MP1R	AGATTCTTGCCATGGGGCAT			
V1F	TGCTTGGAGAGATTTATCAGGGC	293	GVA	Goszczynski and Habili 2012
V2R	GCCTTATCCCAACCCAG			
V3F	TTCAGATTCATGGAGAG			
V4R	CCACCGGTGTAGCTGTA			

minlen: 70), where the low quality sequences regions and ilumina universal adapter sequences were removed. Genome sequences of *Vitis cinerea* x *Vitis riparia* genome strain: BoeWGS1.0 (GENOME ASSEMBLY: GVA_001282645.1), *Vitis aestivalis* cultivar: Norton (GENOME ASSEMBLY: GCA_001562795.1), *Vitis vinifera* cultivar PN40024 (GENOME ASSEMBLY: GCF_000003745.3), (GENOME ASSEMBLY: GCA_004353265), *Vitis coignetiae* chloroplast USDA: NCGR:DVI (NCBI accession: LC333363) and *Vitis coignetiae* voucher Wen_12872 (NCBI accession: MG664829) downloaded from the NCBI database. Using the reference genome assembler: Bowtie2 version 2.3.4, an index reference genome database was constructed with the bowtie2-build function. Host sequences were removed by mapping the trimmed data against the reference genome database and sequence reads that did not map to the reference genomes were collected for meta-assembly. The meta-assembly was done using the metaSPAdes pipeline within the de novo assembler: SPAdes version 3.11.1. Three meta-assemblies were done using 3 different k-mers (K21, K33, K55) for assembly optimisation, the best assembly was used as the final output from the *de novo* assembler. The contigs were then identified using Blast version 2.6.0 against the NCBI nt/nr database. The reference genomes of grapevine virus A (GVA) that were identified in this study (Tab. 1) are deposited in GenBank/EMBL database with the following accession numbers: GVA genetic variants P163-M5 (DQ855082), GTR1-1 (DQ787959) and P163-1(DQ855088).

Results and Discussion

In 2002, the experiment of mealybug transmission of SD from four sources of this disease to SD-free grapevines 'Merlot' was conducted. The SD sources included SD-positive grapevine 'Cinsaut Blanc' clone P163/12. The results were reported in an abstract at the 14th ICVG Meeting in Italy (Goszczynski and Jooste 2003a). In this experiment of transmission of SD from 'Cinsaut Blanc' P163/12 to 'Merlot', three months following the spraying of mealybug-infested recipient 'Merlot' plants with pesticide, some new growing canes remained immature and green, and have not changed since (Fig. 1). Cross sections showed typical SD symptoms: underdeveloped xylem and enlarged phloem tissues. After seven months of observation all these plants were pruned, dsRNA was isolated and tested using GVA- and GLRaV-3 -specific RT-PCRs based on primers pairs H7038/C7273 and H420/C629 respectively. The results showed that all plants of 'Merlot' with symptoms of SD transmitted from 'Cinsaut Blanc' P163/12, were clearly infected with GVA (Fig. 2). SD-negative grapevines did not contain this virus or, estimating from intensity of staining of PCR products, it was at relatively low titres. In the case of 'Merlot' plant 6, only one cane (6b) exhibited SD symptoms and, in comparison with the healthy- looking cane (6a) it was clearly infected with GVA at a higher titre (Fig. 2). Thus, the results suggested a correlation between SD and the presence of GVA. This was not the case in GLRaV-3. The virus was clearly present in most of SD-affected as well as in SD-negative 'Merlot' plants (Fig. 2).

In 2013, selected dsRNA, the RT-PCR results of which are shown in Fig. 2, were re-tested using newly designed GVA-specific primers MP2F, MP1R (Goszczynski 2007) and V1F, V2R, V3F, V4R (Goszczynski and Habili 2012). Cloned amplicons of RT-PCR based on broad GVA variant spectrum primers MP2F/MP1R, SSCP of clones and sequencing of selected clones, clearly showed that 'Cinsaut Blanc' P163/12, the source of SD, and SD-affected 'Merlot' plants from mealybug and grafting experiments are infected with P163-M5 (group II) and P163-1 (group III) variants. This confirmed results obtained earlier (Goszczynski and Jooste 2003b). In canes of 'Merlot' plant 6, which were SD-negative (6a) and SD-affected (6b), respectively, only P163-1 and P163-M5 variants were detected (Fig. 3B). Genetic heterogeneity of fifty-two randomly selected clones of GVA sequences per each cane were investigated using the SSCP technique. The results suggested that the GVA variants of 'Cinsaut Blanc' P163/12 were separated in different canes of the 'Merlot' plant 6. The identity of GVA variants was confirmed by obtained sequence data. The SSCP technique proved to be very useful for rapid investigation of hetero-

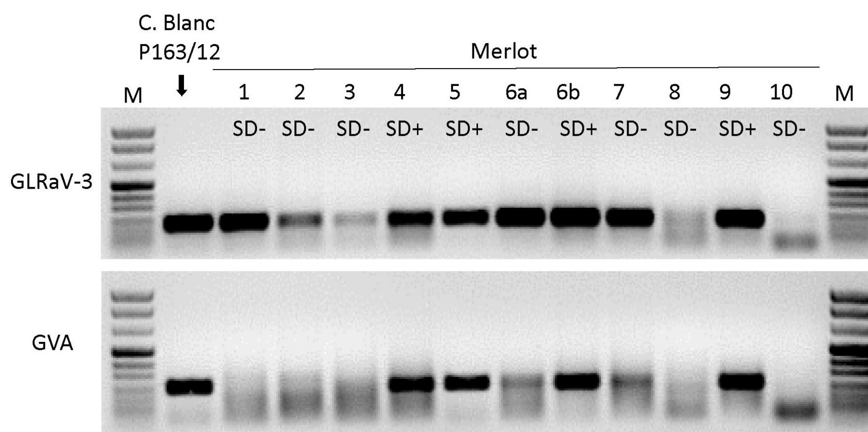

Fig. 2: RT-PCR detection of GLRaV-3 and GVA in SD-positive 'Cinsaut Blanc' clone P163/12 and in SD-free and SD-affected canes of 'Merlot' plants 7 months after transmission of the disease by mealybug *P. ficus*. Primers pairs H420/C629 and H7038/C7273, respectively, were used.

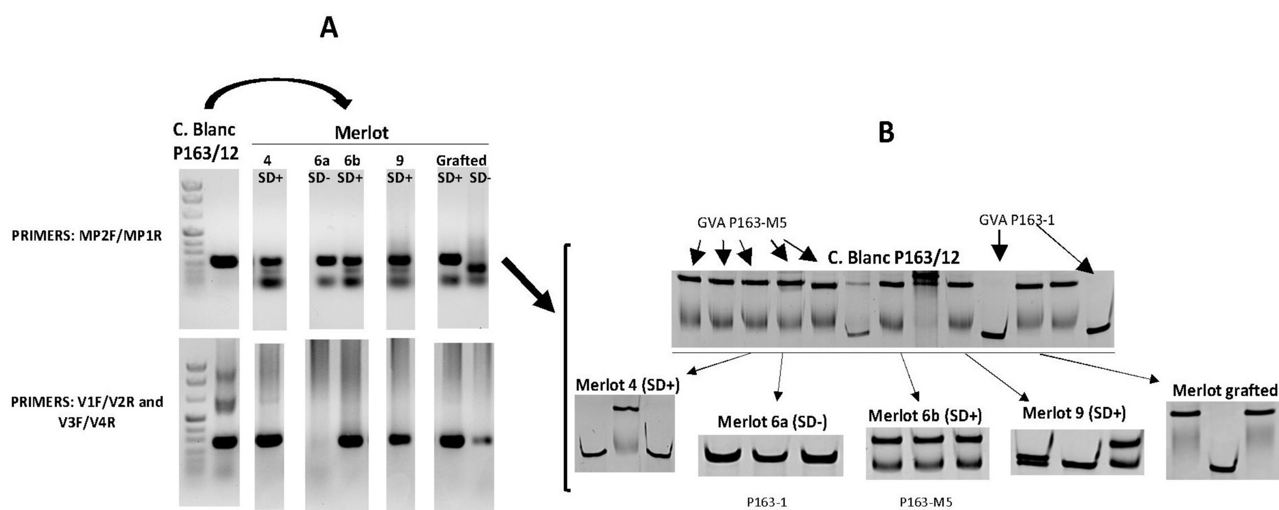

Fig. 3: (**A**) RT-PCR detection of genetic variants of GVA in SD-source 'Cinsaut Blanc' P163/12 and transmitted to 'Merlot' by mealybugs (plants 4, 6 and 9) and grafting, and (**B**) SSCP analysis of heterogeneity of GVA sequences of PCR amplicons obtained in RT-PCR based on MP2F/MP1R primers.

geneity of GVA. Noticeable difference in the SSCP pattern of P163-M5 from 'Merlot' plants 4, 6b and 9 (Fig 3B) was not a surprise because the characteristic SSCP pattern of P163-M5 can drastically change after only 1 nt substitution (GOSZCZYNSKI 2007). In addition, it appears that the "smear" effect of P163-M5 PCR amplicons in PAGE electrophoresis (see Fig. 3B), which is also observed in other members of GVA variants of group II (GOSZCZYNSKI 2007), depends on a batch of ammonium persulfate (APS) used for preparing of polyacrylamide gels (GOSZCZYNSKI, not published). It indicates that, in general, the SSCP technique cannot be used for identification of genetic variants of viruses if not supported by sequence data. The convenience of the SD transmission model used in this study is that 'Cinsaut Blanc' P163/12, the source of SD, is infected only with GVA variants of group II and III. The separation of GVA variants in canes of 'Merlot' plant 6 exhibiting different SD status was confirmed by the results of RT-nested PCR based on V1F-V4R primers, which amplified only GVA variants of group II in the 'Cinsaut Blanc'/'Merlot' model (Fig. 3A). Negative or relatively weak PCR amplicons obtained in RT-nested PCR based on V1F-V4R primers for canes free of SD symptoms, shown in Fig. 3A, clearly suggested that P163-M5 variant is needed to induce this disease. However, owing to the use of virus-specific primers in this study, the results were limited to only two viruses, GVA and GLRaV-3.

To investigate the possible presence of other mealybug-transmissible viruses in 'Cinsaut Blanc' P163/12, the dsRNA isolated from this grapevine was RT-PCR amplified using random hexamers P1-6N (see Material and Methods) as primer, and the amplicons were analysed using HTS. The results firmly confirmed that the grapevine was infected with GLRaV-3 and GVA. The data also suggested the presence of a *Vitivirus*, grapevine virus E (GVE) (Tab. 1). No other plant viruses were detected. In a further study, the same approach (HTS analysis of amplicons of dsRNA generated in RT-PCR based on random hexamers as primer) was applied in the study of the virus status of grapevines used in an experiment to transmit SD from 'Cinsaut Blanc' P163/12, using mealybugs (SD-affected 'Merlot' plant 9) and grafting ('Merlot' and 'Shiraz' plants with SD-affected and SD-free canes). HTS data revealed the presence of GLRaV-3 and

GVA in all investigated plants. Surprisingly, GVE was also easily detected in grafted 'Shiraz' and 'Merlot' exhibiting SD symptoms, suggesting the involvement of this virus in SD. However, the virus was not detected at all in SD-affected 'Merlot' 9 plants from the mealybug transmission experiment of SD (Tab. 1), indicating that GVE is not needed to induce the disease. Although the question whether GVE plays a role in the development of SD remains to be investigated, it is possible that the relatively high titre of this virus in SD-affected tissues is just a result of intensive amplification of GVE in abnormally developed grapevine cells. Again, except for GLRaV-3, GVA and in some cases GVE, no other plant viruses were detected. This suggests that GLRaV-3 and GVA transmitted from 'Cinsaut Blanc' P163/12 to 'Shiraz' and 'Merlot', induce SD. The virus status results should be reliable since the application of RT-PCR based on random hexamers ensures unspecific amplification of all viruses that produce dsRNA.

HTS data revealed a variable populations of genetic variants of GLRaV-3 and GVA. Although a detailed analysis of variant populations was not pursued, unlike GLRaV-3, HTS data for GVA strongly pointed towards an association between GVA-M5 and SD. The variant was detected only in SD-affected grapevines (Tab. 2). In conclusion, GVA-specific RT-PCR techniques, shown in Fig 3, along with HTS data support the earlier hypothesis that GVA-M5 variant is a key virus inducing SD when the disease is transmitted from 'Cinsaut Blanc' P163/12 to SD-susceptible 'Shiraz' or 'Merlot'. After this investigation was concluded, the genome sequence of GVA-M5 variant from SD-affected 'Merlot', named as GVA M5v, was deposited in GenBank/EMBL database with accession number MK982553. The sequence is slightly different from GVA-M5 from *N. benthamiana*. Comparison of these sequences revealed 93 nucleotide substitutions, distributed in protein-coding regions equally along the genome of the virus. The substitutions led to 25 amino acid substitutions, of which 10 are of different charge and 3 of different polarity. Most of these meaningful substitutions are located in the replicase gene of the virus (not shown). The substitutions are most likely the result of GVA host adaptation. Recently cDNA clones of GVA-M5 (Goszczynski 2015) and GVA M5v (Goszczynski, unpubl.) were constructed. Both clones are biologically active in *N. benthamiana*.

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Table 2

Results of HTS analysis of virus sequences amplified from grapevines with various SD status using isolated dsRNA and random hexamers based RT-PCR. Grapevines originated from transmission of viruses from SD-source 'Cinsaut Blanc' clone P163/12 to SD-free 'Merlot' and 'Shiraz' by mealybugs or grafting

Grapevine	SD transmission	SD status of canes	GLRaV-3	GVA			GVE
Cinsaut Blanc P163/12	SD source	SD- positive	+	**P163-M5** (II)[a]	3311/678[b]	2248-5558/99.2[c]	+[d]
				P163-1 (III)	2322/306	4784-7083/98.7	
				GTR1-1 (III)	2002/862	268-2269/92.7	
Merlot pl. 9	mealybugs	SD-affected	+	P163-1 (III)	2690/258	4429-7118/98.5	-
				P163-M5 (II)	2171/986	124-2220/98.9	
				GTR1-1 (III)	659/2088	2179-2835/92.0	
Merlot	grafting	SD- affected	+	P163-1 (III)	3033/1744	288-3033/99.0	+
				P163-M5 (II)	1408/885	109-1515/99.2	
				GTR1-1 (III)	1067/574	5033-6099/99.2	
		Free of SD symptoms	+	Not detected	NA		-
Shiraz	grafting	SD-affected	+	P163-1 (III)	3287/103	3601-6845/94.7	
				P163-M5 (II)	2044/465	121-2164/99.4	+
				GTR1-1 (III)	1273/756	58-1317/93.4	
		Free of SD symptoms	+	GTR1-1 (III)	1481/1176	5788-7250/96.8	+
				P163-1 (III)	489/1828	3839-4316/93.3	

[a] Name of virus variant and phylogenetic group (in brackets) of GVA according to Goszczynski and Jooste (2003). For GenBank/EMBL accession numbers of variants see Material and Methods.

[b] Maximum length of contigs that were assembled/coverage. The contig coverage was calculated as follow: C = LN/G, where C, L, N and G stands for coverage, read length, number of reads mapped to and contig length.

[c] Genome location of contigs and % nt similarity with respected sequences of GVA variants deposited in GenBank/EMBL database.

[d] The values for maximum contig assembled/coverage for GVE were respectively (from top to bottom) 354/1.1, 1398/1851, 7305/41 and 231/1.3.

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