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Single-strand conformation polymorphism (SSCP), cloning and sequencing reveals two major groups of divergent molecular variants of grapevine leafroll-associated virus 3 (GLRaV-3)

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Summary

The SSCP and RE/SSCP analysis of ORF5, ORF6 and ORF7 of 25 local and overseas isolates of GLRaV-3 showed only two kinds of distinct SSCP profiles for each of these genomic regions. It suggested low molecular variability of the virus. Fragments of the 5'UTR + ORF1a, 3' terminal part of ORF1a, ORF4, ORF5, ORF6 and ORF7 of three isolates, representing distinct SSCP profiles, were cloned and sequenced. Results revealed that although the 3'terminal half of the genome (ORF4-7) and the sequence located in the 3' terminal part of ORF1a were relatively similar among isolates (91.3-96.2 % nt identity), their 5'terminal parts (88 nt of 5'UTR and adjacent first 329 nt of ORF1a) were clearly divergent (81.6-81.8 % nt identity). Analysis of this divergent part of GLRaV-3 for an additional 11 isolates showed that they cluster in two distinct molecular groups, sharing 94.7-99.7 % and 80.8-85.1 % nt identity within and between groups respectively. The correlation between the molecular groups and SSCP profiles of the 209 nt fragment of ORF5 of GLRaV-3 strongly suggests that SSCP analysis of this easily RT-PCR amplified region can be used for rapid identification of divergent molecular variants of the virus in field-collected grapevine samples.

K e y w o r d s : GLRaV-3, divergent variants, SSCP, cloning and sequencing.

Introduction

Grapevine leafroll-associated virus 3 (GLRaV-3) is the type member of the *Ampelovirus* genus (Martelli *et al*. 2002). The virus has flexuous particles of about 1800 nm long, containing positive-sense single stranded RNA, of 17,919 nucleotides. Its relatively large genome is organized into 13 open reading frames (ORF1-13) (Ling *et al*. 2004). The virus is common in vineyards worldwide. It is transmitted between grapevines by many species of pseudococcid mealybugs (*Pseudococcus* spp., *Planococcus* spp) and scale insect (*Pulvinaria vitis*) (Gugerli 2003). The consistent association of GLRaV-3 with leafroll disease of grapevines prompted researchers to identify GLRaV-3 as a genuine agent of this economically most important viral disease (Boscia *et al*. 1995). The disease delays ripening of grapevine berries, decreases the accumulation of sugar and ultimately influences the quality of the vine. Despite the negative impact of GLRaV-3 on grapevine industries worldwide, knowledge on the variability of the virus which is essential for developing effective control of the virus in vineyards, is surprisingly poor. There are no reports on the subject except that by Turturo *et al*. (2003). In a short abstract, published in an ICVG Proceedings, they focus, however, on variability within isolates, and little consideration is given to variability between isolates of the virus. Here we report the finding of two major groups of clearly divergent molecular variants of GLRaV-3.

Materials and Methods

Isolates of GLRaV-3 used in this study are presented in Tab. 1. South African isolates were collected from local vine-

T a b l e 1

GLRaV-3 isolates used in this study

GLRaV-3 isolates	Grapevine cultivar	Origin
595	Pinotage	S.A.
612	Cape Riesling	S.A.
614	Cape Riesling	S.A.
616	Shiraz	S.A.
619	C. Sauvignon	S.A.
621	C. Sauvignon	S.A.
623	Ruby Cabernet	S.A.
625	Ruby Cabernet	S.A.
626	Cape Riesling	S.A.
627	LN33	S.A.
628	LN33	S.A.
OV3	Pais	Chile
OV4	Sauvignon Blanc	France
OV6	Weisser Riesling Niederhausen	Germany
OV7	Pinot Blanc Diedesfeld	Germany
OV8	Huxelrebe	Germany
OV10	A. Koudsi x Berlandieri	Israel
OV13	Ruggeri	Italy
OV15	Conegliano	Italy
OV16	C. Sauvignon	Portugal
OV18	Azal Branco	Portugal
OV20	Pinot Noir	Switzerland
OV21	Merlot	Switzerland

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yards near Rawsonville and Badsberg, Western Cape. The overseas isolates were received from the Directorate Plant Production, Health and Quality, Stellenbosch. Isolation of dsRNA, RT-PCR, SSCP, RE/SSCP, cloning, sequencing and computer-assisted sequence analysis were carried out as described by Goszczynski and Jooste (2002). For the SSCP analysis, the PCR products of expected sizes were purified from low-melting agarose using a Wizard PCR Preps DNA Purification System (Promega), then denaturized and electrophoresed in 12 or 15 % polyacrylamide (29.2 % acrylamide/ 0.8 % bis-acrymide) gels. Negative images of EtBr-stained gels are shown. In RE/SSCP analysis, the 1015 bp and 572 bp products of PCR amplification of ORF6 and part of ORF7 were digested with a mixture of equal volumes of the restriction enzymes EcoRI, BglI, SpeI and SspI or Csp45I and XmnI (all from Promega), respectively. The virus-specific oligonucleotide primers used in the RT-PCR are shown in Tab. 2. All primers, except H420/C629 (Mac Kenzie 1997) and SLR3F/ SLR3R (S. Mazumdar, Cornell University, USA), were designed in our laboratory using NCBI nucleotide sequence data (NC004667, AF037268) deposited by Ling (Ling *et al*. 2004). RT-PCR amplification of the 417 nt 5'terminal part of GLRaV-3 containing 88 nt of 5'UTR and adjacent 329 nt of ORF1a was carried out as described by Aguilar *et al*. (2003). Although initially the whole 5'UTR of the virus was targeted for amplification using poly T primer (PdT) and virus-specific reverse primer (GL3.487R) located in adjacent ORF1a, only part of the 5'UTR was successfully amplified along with the predicted ORF1a fragment. As amplification was consistent, we did not try to amplify the whole 5'UTR, but instead used established amplification conditions for further analysis. Cloning of the PCR- amplified fragments was carried out using a pGEM-T Easy cloning system (Promega). At least 3 clones were sequenced in both directions for all isolates. The 417 nt sequence 5' terminal part of GLRaV-3 isolate 623 was deposited in the GenBank/EMBL database (accession number AY704412).

Results and Discussion

The 209 bp DNA fragment complementary to the sequence of ORF5 of GLRaV-3 was efficiently RT-PCR amplified from all 25 virus-infected grapevines used in this study.

The consistently large amount of PCR product rendered this genome fragment of the virus ideal for SSCP analysis. The analysis revealed that 22 isolates of GLRaV-3 were characterized by two profiles; comprising two either closely or widely separated bands (SSCP groups I and II, respectively) (Fig. 1). The remaining three isolates (OV6, OV15 and OV18) revealed profiles only slightly different from those of the two major SSCP groups. The RE/SSCP analysis of 1015 bp and 572 bp DNA fragments, complementary to whole sequences of ORF6 plus adjacent 70 nt of ORF7 and part of ORF7, respectively, also displayed two distinct profiles for each genomic region (Fig. 2). The fragments were successfully amplified in sufficient amounts (strongly EtBr-stained DNA bands) for the analysis for only some (10 and 24, respectively) isolates of the virus. Comparison between isolates of the RE/SSCP profiles of the 1015 bp and 572 bp fragments suggested that isolates 616 and OV10 comprised two divergent variants (Fig. 2). Detailed analysis of isolate 616 using cloning and sequencing of the 1015 bp fragment, which encodes the whole capsid protein of GLRaV-3 (Ling *et al*. 2004), confirmed that and revealed that the divergent variants of this isolate shared 95 % nt identity in this genomic region. Comparison of the predicted amino acid sequences of capsid proteins revealed significant divergences

Table 2

Oligonucleotide primers used in this study

Primer	Sequence	Product size (bp)	Genomic position of amplified sequence
GL3.487R	5' GTTGCGGAAGTTGAGGTCCTT 3'	417	5'UTR+ORF1a (70-487)
PdT	5' GCCGCGCATCCAAGCTTTTTTTTTTTTTT 3'		
GL3.1F	5' AACCAGCAACCACAGAGCCAA 3'	675	ORF1a (5313-5988)
GL3.1R	5' TCGTCTTACCGCCACCTGGG 3'		
GL3.5F	5' CGACGAACTGGACGCAATCG 5'	526	ORF4 (10946-11472)
GL3.5R	5' CAGACACCGTTCCGTCCGTG 3'		
H420	5' GATTTAAGCGCGTTTTTCAGGAC 3'	209	ORF5 (12013-12122)
C629	5' CGGCACGATCGTACTTTCTAA 3'		
GL3.7F	5' CGCCACAGCAGCTTTGGCT 3'	478	ORF6 (13448-13926)
GL3.7R	5' CCGTCGAAGCAGCAGCTTGT 3'		
SLR3F	5' GGCATTTGAACTGAAATT 3'	1015	ORF6+ORF7 (13271-14286)
SLR3R	5' GTATAAGCTCCCATGAAT 3'		
GL3.8F	5' CGTCCGACGTAACGGAATTGA 3'	572	ORF7 (15072-15644)
GL3.8R	5' TCGCATAGCGCAAGCCATG 3'		

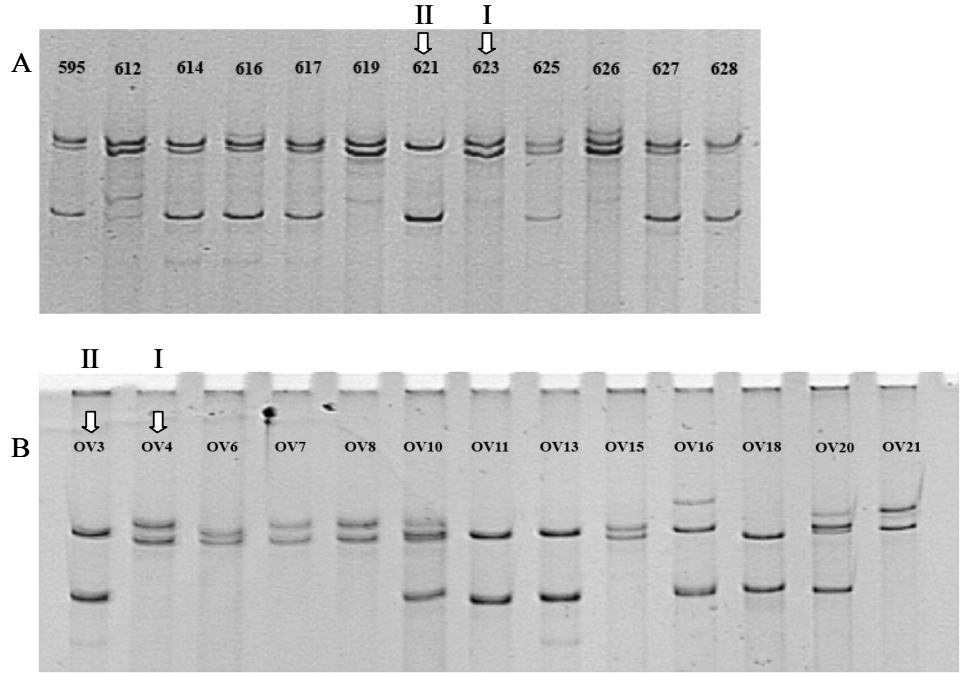

Fig. 1: SSCP profiles of the 209 nt fragment of ORF5 of various isolates of GLRaV-3. Arrows indicate profiles typical of SSCP groups I and II among local (A) and overseas (B) virus isolates.

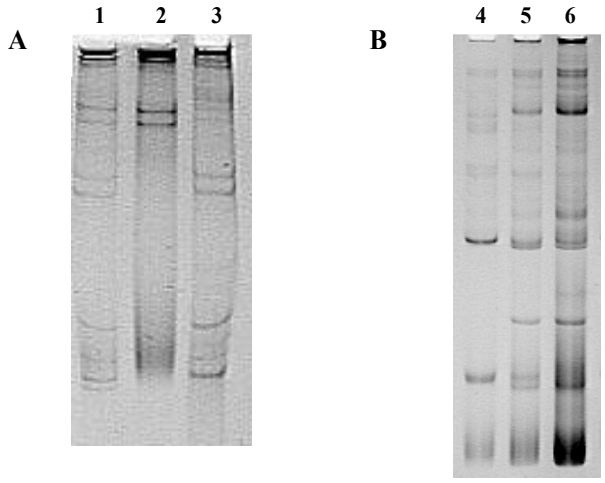

Fig. 2: RE/SSCP profiles of 1015 nt (ORF6 + part of ORF7) (A) and 572 nt (part of ORF7) (B) of GLRaV-3 isolates 616 (1), 623 (2), 628 (3), OV8 (4), OV10 (5) and OV13 (6).

between variants, which included substitutions of hydrophilic with hydrophobic amino acids (Fig. 3).

The presence of two kinds of SSCP profiles in ORF5, ORF6 and ORF7 suggested that the molecular variability of the virus is low and the isolates probably cluster into two major molecular groups. To investigate this hypothesis, two isolates (623 and 621), showing distinct SSCP profiles of ORF5 (SSCP group I and II, respectively), were selected for molecular analysis. RT-PCR amplified genomic fragments comprising parts of 5'UTR+ORF1a (417 nt), the 3' terminal part of ORF1a (675 nt), ORF 4 (526 nt), ORF5 (209 nt), ORF6 (478 nt) and ORF7 (572 nt) (Tab. 1) were cloned and sequenced. The sequence data revealed that the isolates share 91.8-96.2 % nt identity in ORF4 - 7. The most and least divergent were fragments of ORF5 and ORF7 (91.8-92.3 % and 96.0-96.2 % nt identity), respectively. Fragments of ORF1a located between 5313-5988 nt showed 91.3 -91.7 % of nt identity between isolates. The fragments of the 3'terminal half of the virus (ORF4 -7) were cloned for the isolate OV15 as well, which had a SSCP profile similar to that of isolate 623 (SSCP group I) (Fig. 1). The isolate shared 98.8 - 100 % nt identity with isolate 623 in all these regions.

Clear divergence between GLRaV-3 isolates was revealed in the 5' terminal part of the virus. The 417 nt fragment, which included 88 nt of 5'UTR and adjacent 329 nt of ORF1a, revealed 81.8 % nt identity between isolates 621 and 623. The isolate OV15 was almost identical to isolate 623 (98.5 % of nt identity) in this region. Divergence between variants was especially clear in the 5'UTR part, in which the isolates shared only 63.6-64.8 % of nt identity. Study of the 417 nt 5' terminal region of GLRaV-3 for an additional 11 isolates showed that all isolates cluster within two divergent molecular groups (I and II), sharing 80.8-85.1 % and 94.7-99.7 % of nt identity, between and within groups, respectively. The sequence of the GLRaV-3 isolate from the USA (NY1) (Ling *et al*. 2004) clustered in molecular group II, sharing 98.7-99.5 % of nt identity with the isolates of this group. The alignment of the 88 nt 5'UTR fragments for all isolates, including the one from the USA, revealed that sequences in this region are uniform among isolates within each group, with some deviation only in 2 clones of isolate OV8 (Fig. 4). The clones OV8-3 and OV8-4, which shared 94.7-96.2 % and 83.8-85.1 % of nt identity in 417 nt of the 5' terminal part with isolates of molecular group I and II, respectively, had a 12 nt fragment in the 5' UTR identical to that of the divergent molecular group II (Fig. 4). Thus the clones may represent variants of GLRaV-3 originated with recombination between divergent variants of the virus. Our analysis clearly showed that isolates OV8 and OV6 comprise two divergent variants of the virus (Fig. 4). Comparison of SSCP profiles of ORF5

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CP616a  AFELKLGQIYEVVPENNLRVRVGDAAQGKFSKASFLKYVKDGTQAELTGIAVVPEKYVFA
CP616b  ------------------------------------------------------------

                      ●●    ● ●
CP616a  TAALATAAQEPPRQPPAQVVEPQETDIGVVPESETLTPNKLVFEKDPDKFLKTMGKGIAL
CP616b  ------------K--TT-----P-A-----------------------------------

                                    ●  ●       ●  ●●
CP616a  DLTGVTHKPKVINEPGKVSVEVAMKINAALMELCKKVMGADDAATKTRFFLYVMQIACTF
CP616b  --------------------------T--V--------S--TT---K------------

CP616a  FTSSSTEFKEFDYIETDDGKKIYAVWVYDCIKQAAASTGYENPVRQYLAYFTPTFITATL
CP616b  ------------------------------------------------------------

CP616a  NGKLVMNEKVMAQHGVPPKFFPYTIDCVRPTYDLFNNDAILAWNLARQQAFRNKTVTADN
CP616b  ------------------------------------------------------------

             ●
CP616a  TLHNVFQLLQKK
CP616b  -----S------
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Fig. 3: Alignment of predicted amino acid sequences of GLRaV-3 capsid proteins of molecular variants of isolate 616. Symbols (●) indicate amino acid substitutions with different polarity.

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OV18       GTTGCGGAAGTTGAGGTCCTTCTTCTTTAGTTCCCCCTTTTTAACCCTTTTAGAAATTTC
OV6b       ------------------------------------------------------------
OV15       ------------------------------------------------------------
626        ------------------------------------------------------------
625        ------------------------------------------------------------
619        ------------------------------------------------------------
612        ------------------------------------------------------------
OV21       ------------------------------------------------------------
623        ---------------------------------------------t--------------
OV8-3      ---tga-ggaagttt-------------------------------------------
OV8-4      ---tga-ggaagttt--------c-----------------------------------
OV8-7      ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
595        ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
621        ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
NY1(USA)   ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
OV13       ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
OV6a       ---tga-ggaagttt-c----------cc--cgt--t-cg-a----a--a-ttct-----
OV3        ---tga-ggaagttt-c----------cc--cgt--t-cg-a--t-a--a-ttct-----

OV18       CTCTTTTTTCAAGTTTTCAAGTTTCGCC
OV6b       -----c----------------------
OV15       -----c----------------------
626        -----c----------------------
625        -----c----------------------
619        -----c----------------------
612        -----c----------------------
OV21       -----c----------------------
623        -----c----------------------
OV8-3      --t--c----------------------
OV8-4      --t--c----------------------
OV8-7      ----cc---t-------t---------t
595        ----cc---t-------t---------t
621        ----cc---t-------t---------t
NY1(USA)   ----cc---t-------t---------t
OV13       ----cc---t-------t---------t
OV6a       ----cc---t-------t-g-------t
OV3        ----cc---t-------t---------t
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Fig. 4: Alignment of 88 nt of the 5'UTR parts of various isolates of GLRaV-3. Not shadowed and shadowed are sequences of virus variants of molecular groups I and II, respectively.

with the divergent molecular groups of GLRaV-3 that we identified clearly showed that the isolates of the single SSCP group belong to one molecular group. This clear-cut correlation suggests that the SSCP technique can be used for rapid identification of divergent molecular variants of the virus. Thus, if sequence data indicate that an isolate comprises two divergent variants of the virus and we observe only one SSCP profile, like in the case of isolates OV6 and OV8, it probably means that variants of a single molecular group dominate in these isolates.

The divergence in the 5' terminal parts of the viral genome has also been found among isolates of *Citrus tristeza virus* (CTV). This virus, like GLRaV-3, is a member of the *Closterovirus* family (Martelli *et al*. 2002). Partial correlation between molecular divergence and differences in pathogenic properties of CTV isolates was found (Ayllon *et al*. 2001). The common mixed infections of citrus plants with divergent molecular variants frequently made the validation of biological traits to a single variant difficult (Lopez *et al*. 1998; Ayllon *et al*. 2001). The study of this virus revealed also that in some cases the population structure of variants in the field was drastically changed after transmission by aphid vectors (Albiach-Marti *et al*. 2000). It suggests that aphids may transmit the molecular variants of CTV with different efficiencies.

The question of whether isolates of the divergent molecular groups of GLRaV-3, described in this paper, differ in biological properties, like their pathogenic effect on grapevines and the efficiency of their transmission by insect vectors, is intriguing. The answer may be important for understanding the epidemiology of the virus.

Note added in proof: A comprehensive article on genetic variability of 45 isolates of GLRaV-3 was published by Turturo *et al.* (J. Genet. Virol. **86** (2005), 217-224). The results indicate limited genetic variability of the virus, which agree with the results presented in this paper.

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