Vitis **41** (2), 77–82 (2002)

The application of single-strand conformation polymorphism (SSCP) technique for the analysis of molecular heterogeneity of grapevine virus A

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Summary

The results of the analysis of grapevine virus A (GVA) isolates by single-strand conformation polymorphism (SSCP) confirm that this technique is very helpful in rapid and relatively low cost preliminary analysis of molecular heterogeneity of viruses. The results clearly show that the reliability of SSCP analysis of GVA depends on oligonucleotide primers for successful RT-PCR amplification of the highest possible number of molecular variants of the virus. Among 7 pairs of GVA-specific primers designed in different laboratories only two, those from Canada (H7038 and C7273) and Switzerland (MP and CPdt), allowed positive RT-PCR amplification of all our isolates of the virus mechanically transmitted from various grapevines to *Nicotiana benthamiana*. With SSCP analysis of 238 bp DNA fragments complementary to part of ORF5 of GVA, produced by RT-PCR using the first pair of primers, we were able to detect 1-35 nt differences between GVA isolates. The DNA fragments, about 986 bp, complementary to part of ORF3 and ORF4, ORF5 and 3'UTR of GVA, produced by RT-PCR using the second pair of primers, were useful for SSCP analysis only after their digestion with the restriction enzyme *Dde*I. The results strongly suggest that SSCP analysis of 238 nt fragment of ORF5 of GVA along with *Dde*I/SSCP analysis of about 986 nt 3' terminal fragment of the virus allow rapid and reliable determination of the number of dominant nt sequence variants of GVA present in a single *N. benthamiana* or grapevine plant.

K e y w o r d s : isolates of grapevine virus A, RT-PCR, SSCP, cloning and sequencing.

Introduction

Grapevine virus A (GVA) is the type member of the *Vitivirus* genus (Martelli *et al*. 1997), characterized by the genome of positive sense single-stranded RNA, of 7349 nucleotides, excluding a poly (A) tail at the 3' terminus, organized into 5 open reading frames (ORF1-5) (Minafra *et al*. 1997). The close association of the virus with Kober stem grooving (KSG), which is one of the 4 economically important grapevine diseases of the rugose wood complex (RW) (Minafra, 2000), was found (Garau *et al*. 1994; Chevalier *et al*. 1995). The isolation of GVA and its transmission back to grapevines (fulfillment of Koch's postulates) is needed to definitely determine the involvement of the virus in the aetiology of KSG. As GVA can be transmitted to herbaceous plants by insect vectors (Boscia *et al*. 1997) or mechanically (Monette *et al.* 1990 a; Goszczynski *et al*. 1996), many isolates of the virus were recovered from grapevines in different laboratories. The reported isolation of GVA variants with distinct pathogenicity for *N. benthamiana* (Monette and James 1990 b), and differentiation of GVA isolates by monoclonal antibodies (Gugerli *et al*. 1990; Boscia *et al*. 1992), or reverse-transcription polymerase chain reaction (RT-PCR) (De Meyer *et al*. 2000), indicate the heterogenic nature of this virus.

The single-strand conformation polymorphism (SSCP) technique, discovered by Orita *et al*. (1989), was found to be very useful for quick, sensitive and relatively inexpensive detection of differences in the nucleotide sequences of closely related virus genomes (nucleotide sequence variants). The basis of the technique is the discovery that even a single nucleotide difference in PCR-amplified DNA sequence can be detected by the different electrophoretic mobility of separated single strands of this DNA in non-denaturing polyacrylamide gels. For SSCP analysis of plant RNA viruses, a targeted fragment of a virus genome is amplified by RT-PCR and the DNA complementary to virus sequence is analysed using a general SSCP procedure (Magome *et al*. 1999).

In this paper we show that the technique is very useful for the rapid determination of the number of dominant sequence variants of GVA in virus-infected herbaceous plants as well as in grapevines.

Material and Methods

G V A i s o l a t e s a n d t h e i r m e c h a n i c a l t r a n s m i s s i o n f r o m g r a p e v i n e s t o *N i c o t i a n a b e n t h a m i a n a* : GVA isolates GTR1-1, GTR1-2 and GTR1-3 used in this study were recovered from a single plant of *Vitis vinifera* L. cv. Shiraz (referred here as Shiraz GTR1), and those of GTG11-1, P163-1, JP98, 92/778 and MSH18-1 from Shiraz (GTG11), Cinsaut Blanc clone P163/12, Waltham Cross, Cabernet Sauvignon and Shiraz (MSH18) grapevines respectively. Grapevines were collected from vine-

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yards near Stellenbosch, South Africa, except the Cinsaut Blanc clone P163/12 and Cabernet Sauvignon, which were received from the Directorate Plant Production, Health and Quality, Stellenbosch.

Mechanical transmissions of GVA were carried out as described by GOSZCZYNSKI *et al*. (1996). For transmission of the virus from grapevines, preparations of viruses partially purified from grapevine leaf petioles (Cinsaut Blanc P163/12, Waltham Cross) or cane phloem were used.

Isolation of dsRNA and reverse transcription polymerase chain reaction (RT-PCR): DsRNA was isolated from virus-infected *N. benthamiana* or grapevines according to the method described by HU *et al*. (1990). 2 ml of the preparation of dsRNA in ethanol were centrifuged in a Sigma 101M microcentrifuge at 15,000 rpm for 20 min and the obtained pellets were resuspended each in 10 µl sterile deionised water and stored at -80 °C until used. 5 µl of the dsRNA preparations were denatured at 99 °C for 5 min, cooled on ice for 2 min, and then briefly centrifuged. One or 4 µl denatured dsRNA were used in 10 µl of reverse transcription reaction, depending on whether dsRNA was isolated from virus-infected *N. benthamiana* or grapevines. 10 µl reverse transcription reactions were prepared from sterile deionised water (3.25 µl or 0.25 µl) plus 2 µl 5 x M-MLV RT reaction buffer (Promega), 1 µl dNTPs (10 mM each), 1 µl each of forward and reverse primers (100 ng·µl^{-1}), 0.25 µl RNasin (30 U·µl^{-1}) (Promega), 0.5 µl M-MLV reverse transcriptase (200 U·µl^{-1}) (Promega). Reverse transcription was performed at 42 °C for 1.5 h. 1 µl RT was used in 10 µl PCR reaction containing 6.075 µl water, 0.125 µl Taq DNA polymerase (5 U·µl^{-1}) (Promega), 1 µl 10 x buffer A, 0.6 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs and 0.5 µl each of forward and reverse primers. Thermal cycling parameters were as follows: 1 cycle of 98 °C for 2 min, 95 °C for 20 s, 65 °C for 1 min 30 s and 72 °C for 3 min; 35 cycles of 94 °C for 20 s, 56 °C for 30 s, 72 °C for 1 min; and a final elongation at 72 °C for 5 min.

Oligonucleotide primers used in RT-PCR are presented in Tab. 1. Primer pairs H587, C995 were designed by MINAFRA *et al*. (1992); those of H7038, C7273 and MP, CPdt by MACKENZIE (1997) and DE MEYER *et al*. (2000), respectively. Other primers used in this study were designed in our laboratory using GVA sequence data deposited in the GenBank by MINAFRA *et al*. (1997) (GenBank accession number X75433), and the DNAMAN program (Lynnon Biosoft 1996).

Single-strand conformation polymorphism (SSCP) analysis: As the presence of primers in the PCR product may affect the SSCP profile (CAI and TOUITOU 1993), the primers were removed from the samples before analysis. PCR-generated DNA fragments were electrophoresed in 1 % low melting point (LM-2) agarose (Hispanagar) containing 0.5 µg·ml^{-1} ethidium bromide (EtBr), in TAE buffer, at 80 V for 1 h at room temperature in the dark. Single DNA bands with the anticipated molecular weights were cut out from gels and the DNA was purified using a Wizard PCR Preps DNA Purification System (Promega), according to the manufacturer's instruction; 10 µl aliquots of purified DNA were stored at -80 °C until used.

For SSCP, 5 µl purified DNA were mixed with 5 µl Bromophenol Blue Loading Solution (Promega) containing 95 % formamide, 0.05 % bromophenol blue and 10 mM NaOH. The mixture was incubated at 99 °C for 10 min, cooled on ice for 2 min and then electrophoresed in 12 or 15 % acrylamide/bis-acrylamide (29.2/0.8), 0.75 mm gels in 0.5 x TBE buffer at 5 °C for 2 h, using Mini-protean II dual slab cell (Bio-Rad). The gels were stained with 0.5 µg·ml^{-1} EtBr for 10 min, washed and photographed using a digital UVP system (Vacutec-Grab-It Version 2.5) and printed using a SONY Digital Graphic Printer UP-D890. Negative images of gels are shown.

Analysis of PCR-amplified DNA fragments by digestion with the restriction enzyme *Dde*I followed by SSCP(RE/SSCP): Restriction enzyme *Dde*I used in this study was selected using GVA sequence data deposited in the GenBank by

Table 1

Oligonucleotide primers used in RT-PCR amplification of GVA

Primers	Sequence	Product size (bp)	Genomic position of amplified sequence of GVA	Reference
GVA 5 F	GGAGATAGTGGCAATGGTCCGA	480	ORF1	GOSZCZYNSKI and JOOSTE, PPRI
GVA 5 R	CATTTGGTGCCCTATACCGTGG			
GVA 6 F	AAAGCATGCCGGAAGGAGTGAC	549	ORF1	GOSZCZYNSKI and JOOSTE, PPRI
GVA 6 R	CGCAAAGCACATCGGGGTATT			
GVA 7 F	CAATGACGTCGCGAGATTGT	664	ORF2 , ORF3	GOSZCZYNSKI and JOOSTE, PPRI
GVA 7 R	TGGATGACGCACTTCTTCGTT			
GVA 8 F	TGGTAGCAACGTTCCACTCGT	695	ORF3	GOSZCZYNSKI and JOOSTE, PPRI
GVA 8 R	TGTCTTCATGTCTTCACCGCA			
H587	GACAAATGGCACACTACG	432	ORF4	MINAFRA *et al*., 1992
C995	AAGCCTGACCTAGTCATCTTGG			
H7038	AGGTCCACGTTTGCTAAG	236	ORF5	MACKENZIE, 1997
C7273	CATCGTCTGAGGTTTCTACTA			
MP	TGCCAGAGGTGTTTGAGACAAT	986	ORF3, ORF4, ORF5, 3'UTR	DE MEYER *et al*., 2000
CPdt	TTTTTGTCTTCGTGTGACAACCT			

MINAFRA *et al*. (1997) and the DNAMAN program (Lynnon Biosoft 1996). According to computer-assisted analysis, RT-PCR amplified 986 bp DNA, complementary to part of ORF3 and ORF4, ORF5 and 3'UTR of GVA (Tab. 1), is cut by *Dde*I to 9 fragments of 130, 188, 42, 146, 98, 78, 184, 38, and 91 bp.

5 µl Wizard-purified DNA were digested with *Dde*I (Promega) in 20 µl reaction mixture containing 12.3 µl sterile, deionised water, 2 µl RE 10 x buffer, 0.2 µl acetylated BSA (10 $\mu g \cdot ml^{-1}$) and 0.5 µl restriction enzyme, at 37 °C for 4 h. Then, 20 µl Bromophenol Blue Loading Solution were added and samples were analysed by SSCP, as described above.

Cloning, sequencing and sequence analyses: DNA fragments amplified by RT-PCR using MP, CPdt primers (Tab. 1), were directly purified using a Wizard PCR Preps DNA purification system (Promega), A-tailed, ligated to pGEM-T Easy Vector (Promega), and then transformed to JM109 High Efficiency Competent cells (Promega) according to the manufacturer's instructions. Transformation cultures were plated onto LB/ampicillin/IPTG/X-Gal plates and white colonies were screened for plasmids with inserts. For nucleotide sequencing, plasmids with expected inserts were purified using QIAprep Spin Miniprep Kit (Qiagen). Selected clones (at least three per GVA isolate) were sequenced in both directions by the DNA Sequencing Service, Department of Molecular and Cell Biology, University of Cape Town. Obtained nucleotide sequence data were analysed using the DNAMAN program (Lynnon Biosoft 1996).

Results and Discussion

RT-PCR detection of GVA transmitted to *N. benthamiana*: Among 7 pairs of primers designed for RT-PCR amplification of different parts of the GVA genome (Tab. 1), the expected product for all isolates of the virus was obtained with only two of them, H7038, C7273 and MP, CPdt (Tab. 2). The results reveal the divergence of nt sequences among GVA isolates, confirming molecular heterogeneity of GVA reported by DE MEYER *et al*. (2000).

SSCP analysis of GVA from *N. benthamiana* and grapevines: As primers H7038 and C7273 ensured highly efficient and consistent RT-PCR amplification of all GVA isolates, they were used in the analysis of this virus by SSCP. The results show that among 8 isolates of GVA in *N. benthamiana,* only isolates GTR1-1, P163-1 and JP98 had similar SSCP profiles. For other isolates, each SSCP profile was unique (Fig. 1), revealing heterogeneity of GVA. The SSCP analysis of more GVA isolates from different grapevine cultivars is necessary to determine whether grouping of isolates according to SSCP profiles is possible.

Interestingly, the result suggests that isolate GTR1-3 contains GVA variants with SSCP profiles identical to that of isolates GTR1-1, and similar to that of isolate GTR1-2 (Figs 1 and 2). These three GVA isolates were recovered from a single grapevine, cv. Shiraz (GTR1). SSCP analysis of GVA from this grapevine strongly suggests that it contains GTR1-1 and GTR1-2 virus variants transmitted to *N. benthamiana*, and that isolate GTR1-3 from *N. benthamiana* contains all variants of the virus detected in the GTR1 grapevine (Fig. 2). A similar SSCP analysis was done for GVA from Shiraz MSH18 and Cinsaut Blanc P163/12 grapevines. SSCP profiles showed more than two bands (Fig. 2), suggesting the

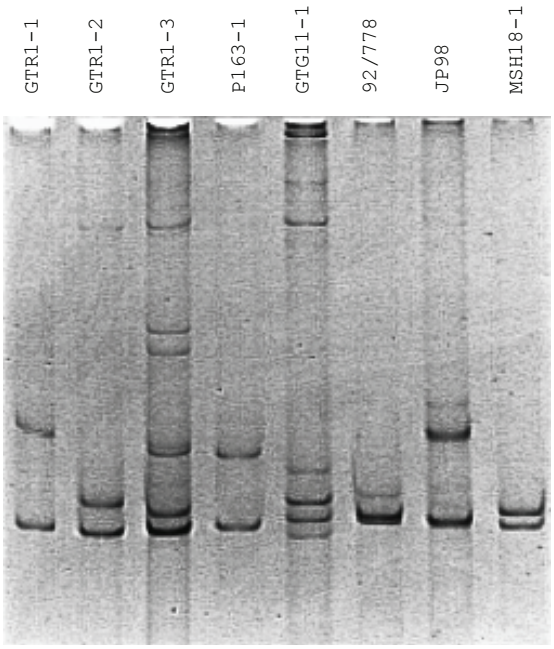

Fig. 1: SSCP analysis of various isolates of GVA established in *N. benthamiana*.

Table 2

Detection of GVA isolates by RT-PCR using different primers

GVA isolate	GVA5 F GVA5 R	GVA6 F GVA6 R	GVA7 F GVA7 R	GVA8 F GVA8 R	H578 C995	H7038 C7273	MP CPdt
GTR1-1	-	-	-	-	-	+	+
GTR1-2	+	+	-	-	+	+	+
GTR1-3	+	+	-	-	+	+	+
P163-1	-	-	-	-	-	+	+
GTG11-1	+	+	+	+	+	+	+
JP98	+	-	-	-	+	+	+
92/778	+	+	+	+	+	+	+
MSH18-1	+	+	+	+	+	+	+

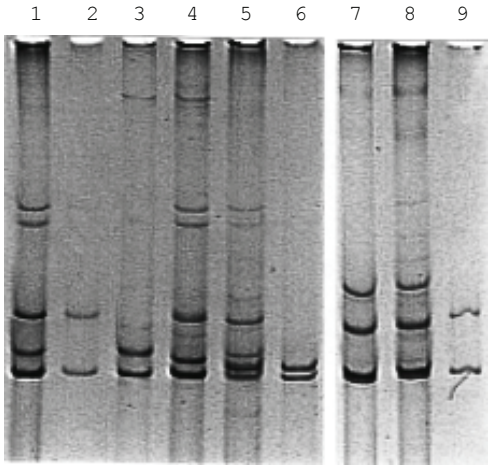

Fig. 2: Comparison of SSCP profiles of GVA isolates from grapevines (Shiraz GTR1; Shiraz MSH18; Cinsaut blanc P163/12, petioles; C. blanc P163/12, cane shavings) and those mechanically transmitted to *N. benthamiana* (GTR1-1; GTR1-2; GTR1-3; MSH18-1; P163-1).

presence of more than one GVA sequence variant in these grapevines. Results indicate that dominant sequence variants in these grapevines were transmitted to *N. benthamiana.* The SSCP profiles for GVA from leaf petioles and cane shavings of Cinsaut Blanc P163/12 were the same, indicating that GVA variants were evenly distributed in this grapevine (Fig. 2).

The presence of different sequence variants of the virus in the same plant is a frequently reported phenomenon (CANDRESSE *et al*. 1995; MAGOME *et al*. 1999; AYLLON *et al*. 2001). Advanced studies with the *Citrus tristeza* virus (CTV) showed that symptoms in *Citrus* induced by this virus depend on the degree of interference between various variants of the virus present in the same plant (MORENO *et al*. 1993; AYLLON *et al*. 2001). Since it is possible that this phenomenon is more common, the method of rapid detection of GVA sequence variants reported here could be very useful for the study of this virus.

Due to the frequent occurrence of the second stable conformations of single DNA strands, which migrate differently in electrophoresis (RUBIO *et al*. 1996), the interpretation of SSCP profiles with more than two bands is sometimes difficult and can lead to wrong conclusions concerning the number of sequence variants present in a single plant. This was the case for isolate GTG11-1. The SSCP profile of this isolate was composed of 4 bands (Fig. 1). Two of these bands were less intensively stained, and the results of further studies (see below) strongly suggest that they represent second stable conformations of single strands of DNA.

Analysis of GVA isolates by RE/SSCP: For SSCP analysis we used RT-PCR amplified DNA complementary to relatively short nucleotide sequence of GVA located in ORF5 (Tab. 1). This fragment comprises only about 3 % of the virus genome. Since a small percentage of the GVA genome was analysed, major errors in the interpretation of the presence of sequence variants of this virus in plants could occur. To test whether more sequence variants of the virus could be detected, we amplified the 3' terminal sequence of each GVA isolate by RT-PCR using primers MP, CPdt (Tab. 1). Amplified DNA, about 986 bp, is complementary to the GVA genome comprising part of ORF3 and ORF4, ORF5 and 3 'UTR. The amplified DNA fragment was not useful for direct SSCP analysis, but after its digestion with restriction enzyme *Dde*I, followed by SSCP, we found that it worked well for the detection of sequence differences between GVA isolates. The comparison of SSCP profiles between isolates strongly suggests that GVA isolate GTR1-3 is composed of sequence variants of isolates GTR1-1 and GTR1-2 (Fig. 3), which confirms results of SSCP analysis of a RT-PCR-amplified nt sequence from ORF5 of these isolates. A clear and limited number of bands in SSCP of part of ORF5 sequence as well as RE/SSCP of about 986 nt fragments of 3' terminal part of genomes of GVA isolates suggests that, except for isolate GTR1-3, isolates GTR1-1, GTR1-2, P163-1, GTG11-1, 92/778, JP98 and MSH18-1 are single sequence variants of the virus. This illustrates that amplification of GVA isolates using primers MP, CPdt and digestion of the PCR product with *Dde*I followed by SSCP analysis is very helpful in determining the number of sequence variants of the virus present in a single plant. The results clearly reveal that the GVA isolate GTR1-3 contained two sequence variants of the virus and the SSCP profile of isolate GTG11-1, composed of the 4 SSCP bands (see Fig. 1), contained second stable conformations of single strands of DNA, thus representing a single sequence variant of the virus.

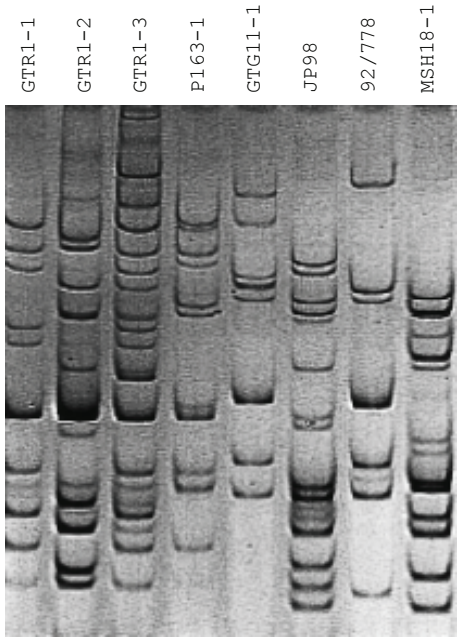

Fig. 3: *Dde*I/SSCP analysis of GVA isolates.

Cloning, sequencing and sequence analyses: The 3' terminal sequences of GVA isolates amplified by RT-PCR using primers MP, CPdt (Tab. 1), were cloned and sequenced. At least three clones were sequenced for each isolate (Goszczynski and Jooste, in prep.). Computer-assisted analysis showed that nt sequences of all our GVA isolates, flanked by sequences complementary to primers H7038 and C7273, analysed earlier by SSCP (Figs 1 and 2), were 2 nt longer (238 nt, including sequence of primers) than

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92/778    AGGTCCACGTATGCTAAGCGTAGGCGCGCTAGGCGCATGAATGTGTGTAAGTGTGGTGCT
MSH18-1   ----------T----------------------------------------------------
GTG11-1   ----------T----------------------------------------------------G
GTR1-2    ----------------A----------------------------------------------
GTR1-3B   ----------------A----------------------------------------------
JP98      -----T-------------------------------------------G----------
GTR1-1    -----T--T--------A------A-G--C----T-----------------------
GTR1-3A   -----T--T--------A------A-G--C----T-----------------------
P163-1    -----T--T--------A------A-G--C----T-----------------------

92/778    ATAATGCACAATAATAAGGATTGTAAATCCAGTAGTATCTCTGGTCACAAACTTGACAGA
MSH18-1   ------------------------G------C--------AA----T-----C------
GTG11-1   --------------------C-----G-----------T--A-----T-----C------
GTR1-2    ----------------------------------------------------------T---
GTR1-3B   ----------------------------------------------------------T---
JP98      ----------------------C-------------------------G--C--T--G
GTR1-1    ---T-------------A-------GG--T----CA-----G-----T-----C--TC--
GTR1-3A   ---T-------------A-------GG--T----CA-----G-----T-----C--TC--
P163-1    ---T-------------A-------GG--T----CA-----G-----T--------TC--

92/778    TTACGGTTCGTGAAAGAGGGAAGAGTAGCCTTAACAGGCGAGACTCCTGTTTACCAAACT
MSH18-1   C----------------------------------G----------------------C
GTG11-1   C----------T-----------------TC-------------------------G----
GTR1-2    --GA-----A-T-----------------TC----G-----------------T-GG--C
GTR1-3B   --GA-----A-T-----------------TC----G-----------------T-GG---
JP98      C-GA-------------------------C-G--G-------------------GG--C
GTR1-1    C-C--------A-----------------C-GGAG-----------------T-G----
GTR1-3A   C-C--------A-----------------C-GGAG-----------------T-G----
P163-1    C-C--------A-----------------C--GAG-----------------T-G----

92/778    TGGATCGAATGGGTTCAGACCGAGTATCATATATATATATTAGAAACCTCAGACGATG
MSH18-1   ---G--A--------G----T-----------------A---------------C-
GTG11-1   ---G--A--------G-------A-----------------------------------
GTR1-2    ---G--A-G------G----------------------C-T---------------
GTR1-3B   ---G--A-G------G----------------------C-T---------------
JP98      ---G--A-G-----GG----T---------------G--C-----------------
GTR1-1    ---G--A-G-----GG-----------------A-------------------T----
GTR1-3A   ---G--A-G-----GG-----------------A-------------------T----
P163-1    ---G--A-G-----AG-----------------A-------------------T----
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Fig. 4: Nucleotide sequence alignment of ORF5 fragments of various isolates of GVA, which were analysed by SSCP.

that of GVA nt sequence by Minafra *et al*. (1997). The alignment of these nt sequences reveals many nt divergencies between isolates of the virus (Fig. 4). The GTR1-3 isolate contains two kinds of sequences: one identical to that of isolate GTR1-1, namely GTR1-3A, and another differing in only 1 nt position from that of GTR1-2, namely as GTR1-3B. The results confirm the heterogenic nature of isolate GTR1-3, shown earlier by SSCP and RE/SSCP (Figs 1-3). The 1 nt difference between sequences GTR1-2 and GTR1-3B is reflected in SSCP profiles (Figs 1 and 2). This illustrates that using SSCP enables us to detect very small differences in nt sequences between variants of GVA. However, comparison of SSCP profiles did not allow us to predict how extensive these differences were. The nt sequence of isolate P163-1, showing a similar SSCP profile to isolate JP98 and GTR1-1 (Fig. 1), differed in 34 and 3 nt positions, respectively, and from isolates with clearly different SSCP profiles (GTG11-1, 92/778, MSH18-1), in 33, 34 and 35 positions (Fig. 4).

The results of SSCP, RE/SSCP and molecular analysis strongly suggest that we isolated and established single sequence variants of GVA in a herbaceous plant. The transmission of some of these GVA variants back to grapevines, would help to better understand the involvement of this virus in grapevine diseases.

Acknowledgement

This work was partially supported by Winetech, South Africa.

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Received November 15, 2001