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FULL RESEARCH PAPER

Genetic variability and population structure of *Grapevine virus A* coat protein gene from naturally infected Italian vines

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Abstract *Grapevine virus A* (GVA) is considered one of the viruses associated with rugose wood (RW), one of the most economically important diseases of grapevine. Thirty-seven GVA isolates collected from grapevine cultivars from Marche (central-eastern Italy), Apulia and Campania (southern Italy), were subjected to molecular characterization. The genetic and population diversity was studied in the coat protein (CP) gene by RT-PCR-RFLP analysis with three restriction enzymes (*Mse*I, *Alu*I, and *Aci*I), and nucleotide sequencing. A new primer pair (CP1F/R) allowing amplification of the whole CP gene (621 bp) was developed. RFLP with *Aci*I yielded the highest number of variants in GVA isolates, showing seven different 'simple' profiles (A, B, C, D, E, F, and G). 'Complex' profiles were also found, and the most common variant combination was A+B in 39% of isolates. The analysis of GVA sequences confirmed the presence of plants infected with more than one GVA variant and suggested that RT-PCR-RFLP is suitable for evaluating population diversity of GVA enabling a screening of different haplotypes. The distribution of RFLP profiles and the phylogenetic analysis were not correlated with the location of infected plants, showing the presence of a GVA population with genetic diversity in the average with those of RNA viruses.

Keywords Cloning and sequencing · Diagnosis · Primer · RT-PCR-RFLP · *Vitis vinifera*

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Introduction

Grapevine is susceptible to several graft-transmissible diseases caused by viruses and virus-like agents. Amongst these diseases, rugose wood (RW) has a high incidence in all viticultural regions of the world and can be considered one of the economically most dangerous diseases of grapevine. *Grapevine virus A* (GVA), with GVB and GVD belongs to the genus *Vitivirus* (Martelli et al. 1997) in the family *Flexiviridae* (Adams et al. 2004). The viral particles are filamentous, 800 nm

long, and the single-stranded (ss) RNA genome is composed of 7,349 nucleotides, excluding a polyA tail at the 3′ terminus, organized in five open reading frames (ORFs) (Minafra et al. 1997). GVA plays a key role in the development of RW disorders (Garau et al. 1994) and it is one of the most common grapevine viruses in the Marche Region (central–eastern Italy), found in about 25% of old plants (Romanazzi et al. 2003). In the same region, Romanazzi et al. (2007) showed that the incidence of GVA in the plants of cvs Verdicchio and Lacrima nera from 2- to 6-year-old vineyards was 35 and 16%, respectively. GVA is a phloem-associated virus (Rosciglione et al. 1983) transmitted semi-persistently (La Notte et al. 1997) by many species of pseudococcid mealybugs (Rosciglione et al. 1983) between grapevines.

The best preventive measure to control grapevine viruses and virus-like diseases is sanitary selection and use of clean propagating material. Therefore, efficient methods to identify healthy source plants remain the most useful way to limit the detrimental impact of viruses in grapevine (Walter and Martelli 1997; Rowhani et al. 2005). The diagnostic tools need to be validated for the heterogenic nature in the virus population, shown by the differences in the pathogenic nature of GVA isolates on *Nicotiana benthamiana* (Goszczynski and Jooste 2003), and further supported by the different reaction of monoclonal antibodies (Boscia et al. 1992) and molecular analysis (Goszczynski and Jooste 2002; Sciancalepore et al. 2006).

The methodologies generally used to identify different variants are: single-stranded conformation polymorphism (SSCP) and restriction fragment length polymorphism analysis (RFLP) (Garcia-Arenal et al. 2001). The use of RT-PCR-RFLP allowed the detection of genetic variability in *Grapevine fanleaf virus* (GFLV) (Naraghi-Arani et al. 2001; Vigne et al. 2004), *Grapevine leafroll-associated virus* 1 (GLRaV-1) (Kominek et al. 2005), and *Grapevine leafroll-associated virus* 2 (GLRaV-2) (Bertazzon and Angelini 2004).

In this paper we present (1) the population structure and genetic variability of GVA isolates from Marche, Apulia and Campania by RT-PCR-RFLP and by sequence analysis of the coat protein region, in order to determine relationships among cultivars and geographic origins, and (2) comparison between the existing nucleic acid-based diagnostic tools and the efficiency of a new primer pair (CP1F/R) in GVA detection.

Materials and methods

Virus sources

Twenty-six GVA isolates were collected in commercial vineyards of Marche whereas eight isolates from Apulia and two from Campania were obtained from a varietal collection of the Department of Plant Protection and Applied Microbiology (DPPMA) of the University of Bari, Italy. Isolate TU32, from Tunisia, provided from the same collection was used as the GVA positive control. All these isolates were analyzed by ELISA with specific antibodies (Agritest, Valenzano, Bari, Italy) for the main grapevine viruses (GVA, GVB, GFLV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7) and by RT-PCR with specific primers for RW agents (GVA, GVB, GVD and GRSPaV) (Minafra and Hadidi 1994; Abou-Ghanem et al. 1997; Zhang et al. 1998; Sciancalepore et al. 2006). Grapevine accessions infected only by GVA were preferentially used for further genetic variability studies. Each GVA isolate was considered as a virus population infecting a single grapevine plant.

Total RNA extraction, cDNA synthesis and RT-PCR amplification

Total nucleic acid (TNA) was extracted from 100 mg of cortical scrapings of dormant cuttings, collected from four different branches of each vine, as described by Foissac et al. (2001). Approximately 500 ng of TNA was denaturated in the presence of 500 ng random primers (Promega, Madison, WI, USA) at 95°C for 5 min. Reverse transcription (RT) was carried out in a 50 μl reaction containing 1× Moloney murine leukemia virus (M-MLV) buffer (Promega), 4.8 mM dithiothreitol, 200 μM each dNTP and 200 U M-MLV (Promega) for 1 h at 42°C. After cDNA synthesis, the reverse transcriptase enzyme was inactivated at 70°C for 10 min. cDNAs were amplified with the primer pairs H7038/C7273 (MacKenzie et al. 1997), H587/C995 (Minafra and Hadidi 1994) and GVA6591F/GVA6906R (Goszczynski and Jooste 2003). Some of these primers are claimed by the authors to be able to detect most of the GVA variants (H7038/C7273), and some those belonging to groups I and II (H587/C995), and to group III (GVA6591F/GVA6906R). The RT-PCR products were visualized on a 1.2% agarose gel in 1× Tris–acetate

buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) after staining with ethidium bromide (0.5 μg ml^{-1}), to confirm the expected size of RT-PCR product and to estimate DNA concentration.

Primer design and RT-PCR-RFLP analysis

Three nucleotide sequences of GVA available in GenBank (accession number NC_003604, AF441234, and AF441235) were used to design a new set of primers: CP1F (5′-TGAAGACAAATGGCACACTACGC-3′) and CP1R (5′-GATGGGTCATCCATCTATATCT-3′) using the software NTI vector version 9.0 (Invitrogen, Life Technologies, Carlsbad, CA, USA), in order to amplify 621 bp, more than the complete coat protein gene, and study the different haplotypes.

A 2.5 μl-aliquot of cDNA product was amplified in 20 μl PCR reaction mixture containing 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer and 0.125 U *Taq* DNA polymerase (Promega). PCR with primer CP1F/R was carried out in a Bio-Rad Cycler (Bio-Rad, Hercules, CA, USA) programmed for an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 45 s and 72°C for 60 s, and a final extension cycle at 72°C for 7 min.

A 6 μl-aliquot of RT-PCR product was digested with 1 U of *Mse*I, *Alu*I, and *Aci*I (New England Biolabs, Beverly, MA, USA) in a 20 μl final volume. The mixture was incubated at 37°C for 4 h and RFLP fragments were separated by electrophoresis using a 10% polyacrilamide gel in 1× Tris–acetate buffer at 15 mA for 3 h at room temperature using a Mini-Protean II polyacrilamide gel system (Bio-Rad). The gel was stained with ethidium bromide and visualized using UV transilluminator.

Amplicons from some GVA isolates (LC4, GVA5, GVA12, LC3, LC2, GVA1, GVA9, LC7) thought to be representative of the profile classes (simple and complex patterns), were ligated into pCR® 2.1-TOPO® vector (Invitrogen) using the TOPO TA cloning kit (Invitrogen) and cloned in *Escherichia coli* DH5α (Sambrook et al. 1989). Five hundred nanograms of plasmid DNA, extracted by QIAprep Spin Miniprep (Qiagen, Hilden, Germany), was digested with 0.5 U *Eco*RI. The 621 bp cloned CP-fragment was purified from the agarose gel by Zimo-Spin Column and digested using the same enzymes (*Mse*I, *Alu*I, and *Aci*I) as previously reported.

Frequency of RT-PCR-RFLP simple patterns was calculated only on cloned RT-PCR fragments, analyzing 10 randomly chosen clones per isolate, to describe the haplotype segregation.

Sequence analysis

Nucleotide sequence of selected cDNA clones (from two to seven for each isolates) was determined by ABI Prism sequencer 377 (Perkin-Elmer, Forest City, CA, USA). Multiple alignments were made using CLUSTAL X version 1.8 (Thompson et al. 1997). MEGA 2.1 (Kumar et al. 2001) was used to estimate nucleotide distance according to the method of Jukes and Cantor (1969) for correction of superimposed substitutions. The same software was useful to calculate phylogenetic relationships according the neighbour-joining method (Saitou and Nei 1987) with 1,000 bootstrap replicates.

Results

The 37 isolates under study were positive to GVA only, when analyzed by ELISA with specific antibody for the major grapevine viruses. Instead, using specific primers for other RW agents (GVB, GVD and GRSPaV), 30 isolates out of 37 had GVA and GRSPaV mixed infections, and no infections of GVB and GVD were detected.

Further molecular analysis was carried out for the 37 GVA isolates. The primer pair CP1F/R was compared with H7038/C7273, H587/C995, and GVA6591F/GVA6906R in order to estimate its diagnostic efficiency in detecting GVA infections in grapevine samples. In particular, CP1F/R amplified 100% of the isolates analyzed, as well as the primer pairs H7038/C7273 and H587/C995, able to amplify isolates belonging to groups I and II. The primer pair GVA6591F/GVA6906R, able to detect the variants belonging to group III, did not amplify any of our isolates.

The 621 bp fragment amplified by CP1F/R was used to study the GVA genetic variability in the CP gene by RT-PCR-RFLP analysis. PCR products from 37 GVA isolates digested with the three endonucleases *Mse*I, *Alu*I, and *Aci*I showed two kinds of restriction pattern in the CP region, designated as ‘simple’ or ‘complex’ pattern. In the ‘simple’ pattern, the sum of the molecular weight of the fragments was the same as the initial fragment (621 bp). In the ‘complex’ pattern, the sum of

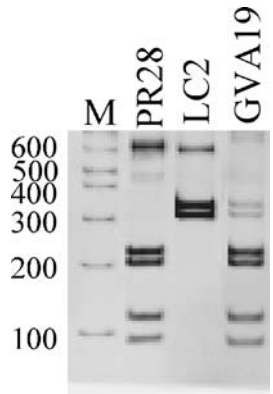

Fig. 1 Example of 'simple' (*PR28* and *LC2*) and 'complex' (*GVA19*) patterns obtained digesting the 621 bp fragment by the endonuclease *Alu*I. The 'complex' pattern originates by the combination of 'simple' patterns

the molecular weight of single fragments exceed the 621 bp fragment (Fig. 1). Figure 2 shows the typical 'simple' banding patterns after the digestion with the restrictions enzymes. The behaviour of all the GVA isolates, when the 621 bp fragment was restricted by the three enzymes, is summarized in Table 1.

In *Mse*I digestion, most of GVA isolates (78%) showed a 'simple' pattern, six isolates (16%) showed a 'complex' pattern whereas in GVA8 and TU32 the fragment was not digested (Table 1). Digestion with *Alu*I produced six different 'simple' profiles (65% of the isolates) while 35% of the isolates showed a 'complex' profiles (Fig. 2). The most recurrent combination was B+C (22%) (Table 1). The digestion with *Aci*I produced seven different 'simple' patterns (42%) (Fig. 2), and the isolates with a RT-PCR-RFLP 'complex' pattern occupied the largest part (58%) versus the 'simple' pattern. Most of the isolates originated from Marche showed a combination of the haplotypes A and B (39%). In a vineyard of Marche, some plants with pattern A, some with pattern B and some others co-infected with the two variants (A+B) were found. Nine out of 10 isolates from Apulia and Campania showed profile G, which was not found among the isolates from Marche.

Some isolates showing 'complex' (LC4, GVA5, GVA12, and LC3) and others with 'simple' patterns (LC2, GVA1, GVA9, and LC7), thought to be representative of the profile classes, were cloned. For each cloned GVA isolate, 10 clones were analyzed by RT-PCR-RFLP with *Aci*I and 47 of them were sequenced (from two to seven clones per isolate).

GVA isolates with the 'simple' pattern showed the same haplotype in all analyzed clones, identical to the one obtained by RT-PCR-RFLP on the initially amplified and uncloned RT-PCR fragment. On the other hand, the clones coming from the isolates with a 'complex' pattern differentiated two haplotypes, one of which was predominant (in the range of 60–80%) (Table 2).

To determinate the accuracy of RT-PCR-RFLP analysis, the genetic diversity of sequenced clones showing the same RT-PCR-RFLP patterns was evaluated according to Jukes and Cantor (1969). GVA isolates LC7 and LC2, showing clones with an identical RT-PCR-RFLP pattern, gave a within-isolate genetic diversity of 0.0032 and 0.0026, respectively. The genetic diversity within single isolates increased in clones with the complex pattern. Indeed, it reached 0.0469 in GVA5 clones with different RT-PCR-RFLP patterns. A different situation was found in the isolate GVA1, where RT-PCR-RFLP patterns of clones was the same but the within-GVA1 genetic diversity (0.0223±0.0042) was comparable to that obtained in LC4 clones (0.0283±0.0041), indicating the presence of different haplotypes (A+C). Twenty-eight nucleotide differences were estimated among sequences of the four clones of the GVA1 isolate. The opposite situation was observed for the GVA12 isolate, that showed a 'complex' pattern (A+B) in the *Aci*I digestion and a low value of within-isolate genetic diversity (0.0060±0.0017).

The between-isolate genetic diversity, calculated according to Jukes and Cantor (1969) is shown in Table 3. In most of the isolates, the genetic diversity was < 0.1. The isolates GVA1 and LC7 behaved differently showing values > 0.1 when compared with all the others. The highest value in pairwise comparison

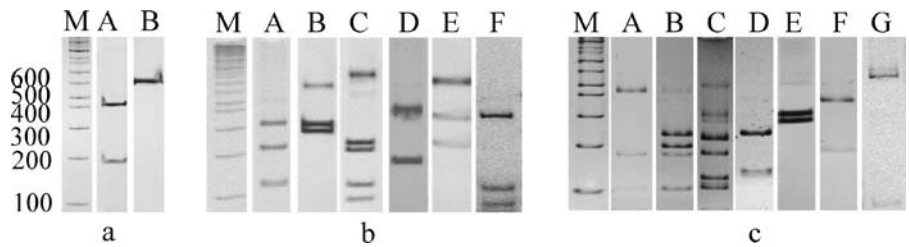

Fig. 2 RT-PCR-RFLP profiles of coat protein gene amplified with the primer pair CP1F/R. Letters above the lanes identify different 'simple' patterns obtained with the endonucleases *Mse*I (**a**), *Alu*I (**b**), and *Aci*I (**c**) respectively; *M* marker 100 bp ladder (Invitrogen)

Table 1 RT-PCR-RFLP analysis in the coat protein gene amplified with the primer pair CP1F/R

Isolate	Cultivar	Location	RT-PCR-RFLP profile		
			*Mse*I	*Alu*I	*Aci*I
GVA1	Verdicchio	Marche, Italy	A	F	D
GVA2	Verdicchio	Marche, Italy	A	Na[a]	**A**+**E**
GVA3	Verdicchio	Marche, Italy	A	**B**+**C**	**A**+**B**
GVA4	Verdicchio	Marche, Italy	A	**B**+**C**	**A**+**B**
GVA5	Verdicchio	Marche, Italy	A	**C**+**E**	**A**+**B**
GVA6	Verdicchio	Marche, Italy	A	**C**+**E**	A
GVA7	Verdicchio	Marche, Italy	A	Na	A
GVA8	Verdicchio	Marche, Italy	B	**C**+**E**	**A**+**B**
GVA9	Verdicchio	Marche, Italy	**C**[b]	Na	**A**+**B**
GVA10	Verdicchio	Marche, Italy	A	Na	**A**+**B**
GVA11	Verdicchio	Marche, Italy	A	A	B
GVA12	Verdicchio	Marche, Italy	A	A	**A**+**B**
GVA13	Verdicchio	Marche, Italy	A	A	A
GVA14	Verdicchio	Marche, Italy	C	Na	**A**+**B**
GVA15	Verdicchio	Marche, Italy	A	E	A
GVA16	Verdicchio	Marche, Italy	A	**C**+**E**	**A**+**B**
GVA17	Verdicchio	Marche, Italy	A	Na	**A**+**B**
GVA18	Verdicchio	Marche, Italy	A	A	**A**+**B**
GVA19	Verdicchio	Marche, Italy	A	**B**+**C**	**A**+**B**
LC2	Lacrima nera	Marche, Italy	A	B	A
LC4	Lacrima nera	Marche, Italy	8	**B**+**C**	**A**+**C**
LC9	Lacrima nera	Marche, Italy	A	**B**+**C**	Na
LC8	Lacrima nera	Marche, Italy	A	D	E
GF	Lacrima nera	Marche, Italy	**C**	C	**B**+**C**
LC7	Lacrima nera	Marche, Italy	A	D	E
LC3	Lacrima nera	Marche, Italy	**C**	C	**C**+**F**
MA24	Malvasia nera	Apulia, Italy	A	F	G
NE22	Negramaro	Apulia, Italy	A	F	G
NE23	Negramaro	Apulia, Italy	A	F	G
PR29	Primitivo	Apulia, Italy	A	F	**A**+**G**
PR28	Primitivo	Apulia, Italy	A	C	G
IM10	Impigno	Apulia, Italy	A	A	G
FI7	Fiano	Apulia, Italy	A	C	A+G
MAR44	Marchione	Apulia, Italy	A	F	G
BI16	Biancolella	Campania, Italy	C	**B**+**C**	**A**+**B**
BI17	Biancolella	Campania, Italy	C	**B**+**C**	**B**+**F**+**G**
TU32	Unknown	Tunisia	B	A	**A**+**B**

[a] Na: sample not analyzed in RT-PCR-RFLP

[b] The bold character indicates a 'complex' pattern

A, B, C, D, E, F, and G are the different patterns obtained by digestion of the amplicon (621 bp) with three endonucleases (*Mse*I, *Alu*I, and *Aci*I)

between isolates was recorded for GVA1 and GVA5 (0.1622). In the phylogenetic analysis of GVA sequences (accessions no. AF441234, AF441235, NC_003604, and AB039841), GVB (AY490135 and AF438410) and a GVD sequence (C. Rosa, UC Davis, CA, USA, personal communication) were included as a reference. In the phylogenetic tree the clones generally clustered according to the typology of RT-PCR-RFLP patterns. The GVA-group I according to Goszczynski and Jooste (2003) can be separated in two subgroups, indicated as Ia and Ib, on the basis of nucleotide similarity. However, all the clones of isolates GVA1 and LC7 were found in two groups genetically distinct between themselves and with the remaining isolates (Fig. 3).

Generally the clones of each isolate clustered in the same group. A different behaviour was shown by isolates GF and LC4, whose clones were localized in two different subgroups. In particular, LC4-5 showing profile A and GF (−8 and −1) showing profile B, both belong to GVA-subgroup Ia, whereas clones GF (−4 and −7) and LC4 (−2, −3, −4, −6, and −7), showing profile C belong to GVA-subgroup Ib. The phyloge-

Table 2 Frequency of RT-PCR-RFLP 'simple' pattern (A, B, C, D, E, and F) obtained by the restriction of the cloned 621 bp-fragment with *Aci*I

Isolate	Clones (*n*)						
	Analyzed	RT-PCR-RFLP pattern					
		A	B	C	D	E	F
GVA1	10	0	0	0	10	0	0
LC7	10	0	0	0	0	10	0
LC2	10	10	0	0	0	0	0
LC4	10	8	0	2	0	0	0
GVA9	10	0	10	0	0	0	0
GF	10	0	2	8	0	0	0
GVA5	10	6	4	0	0	0	0
GVA12	10	2	8	0	0	0	0
LC3	10	0	0	8	0	0	2

netic tree generated from the amino acid sequences revealed essentially the same groups as described for nucleotide data (data not shown).

Discussion

Plant RNA viruses have a great potential for genetic variability due to the absence of proofreading activity in RNA replicase (Garcia-Arenal et al. 2001). Therefore, the primer pair CP1F/R was designed based on the conserved region found on three GVA isolates, whose genome sequences were available, in order to exclude the bias due to possible sequence variants. Moreover, the GVA isolates were amplified with three other primer pairs H7038/C7273, H587/C995, and GVA6591F/GVA6906R, able to detect variants belonging to different groups (I and II, and group III, respectively). Comparing the amplification results of 37 GVA isolates it is possible to conclude that all isolates analyzed belong to groups I and II (Goszczynski and Jooste 2003).

RT-PCR-RFLP proved to be a useful technique to estimate genetic diversity within the GVA population in vineyards and to screen different haplotypes. The majority of GVA isolates, when analyzed with *Aci*I, gave a 'complex' pattern and the most frequent variant combination was A+B, which was present in 39% of GVA isolates from Marche. We hypothesize that the high percentage of plants showing mixed infections (harbouring more than one GVA variant) could mainly result from the initial sanitary status of propagation material (that could be already infected) and the repeated GVA inoculations by mealybugs.

In the isolates generally showing a 'complex' profile, we found that the intensity of DNA fragments in RT-PCR-RFLP profiles reflects the proportion of RNA variants within a GVA isolate. This evidence is supported by the frequency values of RT-PCR-RFLP patterns in the 10 GVA clones, obtained with endonuclease *Aci*I, in which one viral variant was predominant. The co-existence of divergent variants which are in equal concentrations in the same host plant is a rare phenomenon (Garcia-Arenal et al. 2001). If this occurs, it may reflect a possible recent infection, as differences in fitness (Roossinck 1997) will probably cause one variant to dominate with time (Magome et al. 1999). The same A+B variant combination obtained by *Aci*I restriction was present in the Tunisian isolate TU32, which is geographically remote from the tested samples.

The GVA clones with the same RT-PCR-RFLP pattern generally gave a low value of intra-isolate diversity and clustered in the same phylogenetic tree. The variability between the sequences of these clones were estimated to be 5 nt difference, at most, in the 621 bp fragment. These data demonstrate that sequences

Table 3 Between-isolate genetic diversity in the coat protein region, calculated according to Jukes and Cantor (1969)

Isolate	GVA1	LC7	LC2	LC4	GVA9	GF	GVA5	GVA12	LC3
GVA1	–	0.0156[a]	0.0158	0.0143	0.0151	0.0143	0.0173	0.0157	0.0154
LC7	0.1458	–	0.0160	0.0149	0.0162	0.0145	0.0169	0.0164	0.0158
LC2	0.1458	0.1376	–	0.0115	0.0078	0.0110	0.0091	0.0092	0.0122
LC4	0.1347	0.1289	0.0809	–	0.0112	0.0050	0.0127	0.0119	0.0069
GVA9	0.1411	0.1491	0.0507	0.0885	–	0.0103	0.0090	0.0073	0.0116
GF	0.1388	0.1277	0.0838	0.0382	0.0896	–	0.0115	0.0108	0.0070
GVA5	0.1622	0.1543	0.0668	0.1058	0.0730	0.1013	–	0.0080	0.0138
GVA12	0.1392	0.1424	0.0530	0.0896	0.0503	0.0866	0.0577	–	0.0116
LC3	0.1481	0.1442	0.0943	0.0492	0.0990	0.0573	0.1193	0.0926	–

[a] Standard error (SE) values

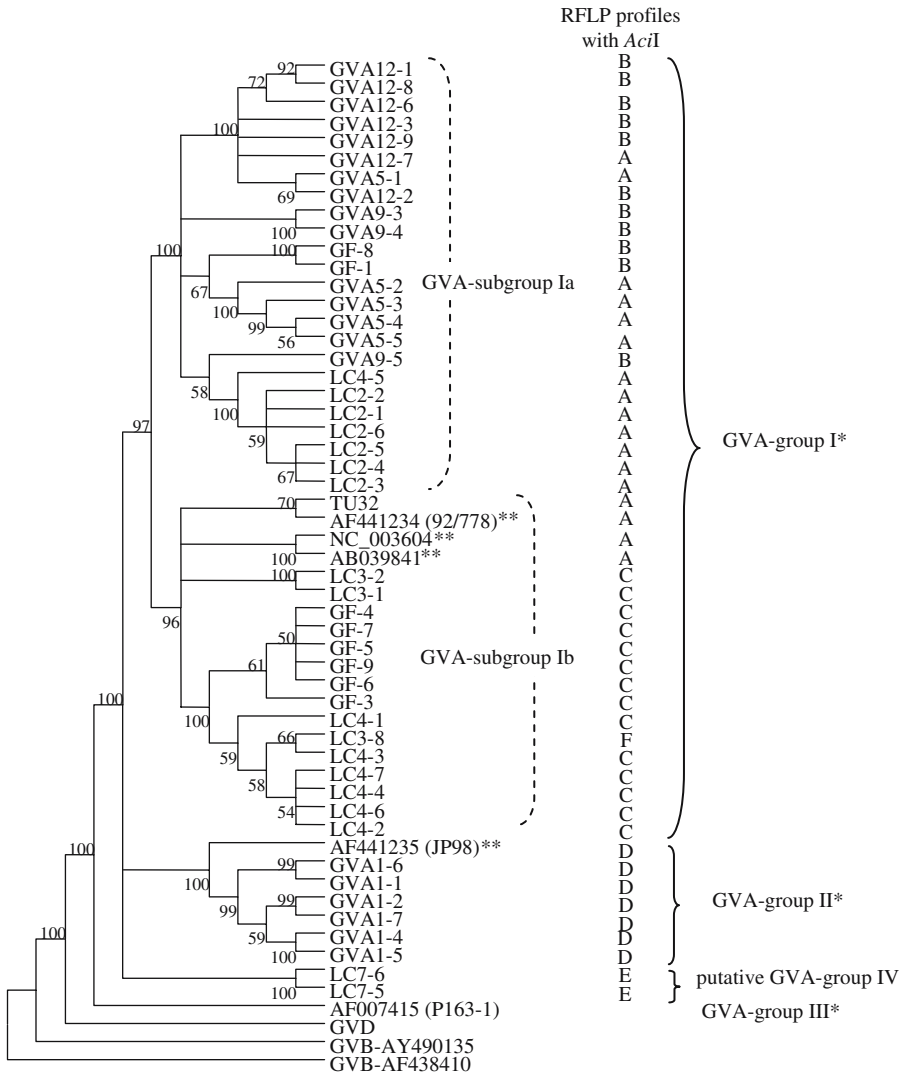

Fig. 3 Phylogenetic tree of genomic CP region constructed by the neighbour-joining method with 1,000 bootstrap replicates, and relationship with the RT-PCR-RFLP pattern obtained by digestion of 621 bp fragment with *Aci*I

*GVA-variant classification according to Goszczynski and Jooste, 2003
**GVA sequences available in GenBank virtually restricted with the software NTI vector version 9.0 (Invitrogen)

related to an isolate are genetically similar and the slight divergence could be ascribed to a '*quasispecies*' nature of GVA, as it happens in all RNA viruses (Domingo et al. 1995). The genetic variability in the GVA coat protein gene (0.1040) was in the range of values obtained in several structural proteins or ORFs of plant viruses (0.0020–0.224; Garcia-Arenal et al. 2001) and those reported for GVA and GVB (Sciancalepore et al. 2006). In this study the majority of isolates analyzed had a genetic diversity <0.10. These data agree with sequence data accumulated during the past 10 years. It seems that in natural populations of plant viruses, genetic stability is the rule rather than the exception (Roossinck 1997).

In previous studies by Goszczynski and Jooste 2002 and 2003, GVA isolates clustered into three groups (I, II, and III) on the basis of nucleotide similarity. In our work the majority of GVA isolates analyzed clustered into one group, including AF441234 (92/778), which is representative of group I. The GVA1 clones (−1, −2, −4, −5, −6, and −7) showing simple pattern 'D' in the restriction with *Aci*I, clustered with AF441235 (JP98) in group II. LC7-clones (−5 and −6), both showing pattern 'E', clustered into a putative

fourth group, that seems to be different from GVA-groups proposed by Goszczynski and Jooste (2003). These clones showed a nucleotide similarity of about 86% both for AF441234 (92/778), representative of group I and AF441235 (JP98) for group II.

For other grapevine viruses such as GLRaV-1 (Little et al. 2001), GRSPaV (Meng et al. 1999), *Grapevine fleck virus* (GFkV) (Shi et al. 2003) and GFLV (Naraghi-Arani et al. 2001; Vigne et al. 2004) mixed infections of viral variants were reported. The presence of multiple variant infections is common in grapevine, and it seems to be related to the long life of the plant, the use of viticulture practices of vegetative propagation and transmission by vectors. This creates ideal conditions for the accumulation of divergent variants of viruses in single plants. The significance of this event is not known. No correlations seem to be present between GVA variants, cultivar and geographic origin. It would be interesting to investigate the possible relationship between the different variant combinations and the range of symptoms.

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