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Genetic variability in *Grapevine virus A* from *Vitis vinifera* L. × *Vitis labrusca* L. in Sichuan, China

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Abstract: *Grapevine virus A* (GVA) has a quasispecies nature and is closely associated with the rugose-wood disease complex of *Vitis vinifera* L. In the different growing regions of table grape in Sichuan, southwest China, the incidence of GVA infection was found to be 16.9% by ELISA detection among 178 grape plants (*Vitis vinifera* L. × *Vitis labrusca* L.). The results of restriction fragment length polymorphism (RFLP) studies on the polymerase chain reaction (PCR) products of a total of 139 plasmids, cloned from 15 GVA isolates, suggested that the GVA isolates contained highly divergent variants. The plasmids from each GVA isolate that yielded different PCR-RFLP profiles were preferentially chosen for sequencing and were designated as variants. Furthermore, a phylogenetic study based on the analysis of the GVA coat protein genes and RNA silencing suppressor genes showed that the 40 variants obtained from the 15 GVA isolates represented 4 clades, designated as molecular groups I, II, III, and IV, respectively. The variant-specific PCR detection results indicated that the parts of the tested grape plants were specifically infected by mild variants.

Key words: *Vitis vinifera* L. × *Vitis labrusca* L., *Grapevine virus A*, PCR-RFLP, cloning and sequencing, RT-PCR

Introduction

Grapevine virus A (GVA) is closely associated with the rugose-wood (RW) disease complex. The RW complex is characterized by modifications of the woody cylinder, including grapevine corky bark, stem grooving, and stem pitting. GVA is spread by mealybug vectors and is considered to be a phloem-associated virus (1). GVA is a member of the genus *Vitivirus*, family *Flexiviridae* (2). The genome of GVA is organized into

5 open reading frames (ORF1-ORF5) that are flanked by 2 short nontranslated sequences at the terminals (3). ORF1 encodes a 194-kDa polypeptide with conserved RNA-dependent RNA polymerase (RdRp) motifs. ORF2 encodes a 19-kDa polypeptide with no significant homology with any known proteins. ORF3 and ORF4 encode a movement protein (*mp*) and a coat protein (*cp*) (4), respectively. Finally, it was well proven that the suppressor of RNA silencing encoded

by ORF5 was involved in pathogenicity as a symptom determinant (5).

Many authors have suggested that reverse transcriptase (RT)-polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis (RFLP analysis of RT-PCR products) is suitable for evaluating population diversity in grapevine viruses (6,7) such as *Grapevine leafroll-associated virus-1* (GLRaV-1) and *Grapevine fanleaf virus* (GFLV). GVA isolates were previously separated into 3 phylogenetic groups based on the overall nucleotide sequence identity in the 3' terminal of the GVA genome (8). An additional putative group IV may infect *Vitis vinifera* L. (9). GVA variants from molecular group II are closely associated with Shiraz disease (10), and variants of molecular group III (designated as GVA mild variants) are common in GVA-infected grapevines that consistently fail to express symptoms of RW disease (11). Universal primers were designed for the RT-PCR detection of all of the molecular GVA variants, and the variant-specific primers GVA6591F and GVA6906R were developed to exclusively detect the mild variants in molecular group III (8).

China is the leading grape-producing country in the world. Grape cultivation is one of the most remunerative farming enterprises in China and the growing land has been estimated at about 644,000 ha, with an annual yield of 8,650,000 t in 2011 (FAO, 2011). Sichuan is a province located in southwestern China. It is one of the most important production areas of table grape in China, with 16,700 ha of growing land and an annual yield of 350,000 t in 2011 (personal communications). While field surveys for RW-related GVA were widely performed in Tunisia (12), Australia (13), South Africa (14), and Germany (15), GenBank contains very limited sequence data for GVA isolated from the hybrids of *Vitis vinifera* L. × *Vitis labrusca* L., widely cultivated plants in China and other countries in East Asia. The objective of this study was to investigate the genetic diversity of GVA isolated from *Vitis vinifera* L. × *Vitis labrusca* L. in growing areas of Sichuan Province using molecular analysis.

Materials and methods

Plant materials

A total of 178 own-rooted plants from 23 varieties of *Vitis vinifera* L. × *Vitis labrusca* L. and 14 grape plants derived from *V. vinifera* L. 'Venus' and 'Red Globe' were randomly collected from 5 growing regions in Sichuan in mid-December. The well-wooded shoots of each plant were taken to the laboratory as soon as possible.

ELISA assessments

Incidences of viral infection were assessed using the enzyme-linked immunosorbent assay (ELISA). Conventional ELISA was performed against GFLV, *Grapevine leafroll-associated virus-2* (GLRaV-2), GLRaV-3, GLRaV-7, GVA, and *Grapevine virus B* (GVB) using commercial kits (AgriTest, Italy), following the manufacturer's instructions. Specifically, ground extracts of bark scrapings were filtered through 2 layers of muslin, and 200- μ L aliquots were placed into duplicate wells. The negative and positive controls supplied by the kit were also included on each plate. Optical density was measured with an ELISA reader (DV990 BV4, GDV, Italy) at an absorbance wavelength of A_{405} nm. Absorbance values that were 3 times greater than those obtained for the mean of the negative control were considered to be ELISA-positive.

RT-PCR-RFLP and plasmid PCR-RFLP

Using the protocol of Foissac et al. (16), the total nucleic acids were extracted from the bark scrapings of the randomly selected 15 (out of the total 178 analyzed) ELISA-positive GVA-infected grape plants. Approximately 500 ng of the DNase-treated total RNA was mixed with 500 ng of hexadeoxyribonucleotide (TAKARA, Japan) and denatured at 95 °C for 5 min. First strand cDNA synthesis was conducted using the *Moloney-Murine leukemia virus* reverse transcriptase enzyme (TAKARA). According to the protocol (17), PCR was conducted using primers designed to specifically amplify part of the GVA *cp* and the full-length silencing suppressor (ORF4-1F and ORF5-1R). The products of the PCR were cloned into plasmid pMD-19T (TAKARA). Finally, the resulting recombinant plasmids were transformed into DH5 α , and at least 6 plasmids from each GVA isolate were then extracted by commercial kits (Tiagen, China).

The restriction enzymes *Alu* I and *Vpak11BI* were selected using Webcutter 2.0 (Max Heiman, USA) to generate appropriate bands of RFLP polymorphism based on known GVA sequences from GenBank. The RT-PCR products of each isolate were subjected to digestion with either *Alu* I or *Vpak11BI* in a 20- μ L reaction mixture (TAKARA), respectively (designated as RT-PCR-RFLP). Next, the restriction fragment mixtures were separated by electrophoresis on a 6% polyacrylamide gel in 1X Tris-acetate buffer at 8 mA for 3 h at room temperature (18). The gel was stained with ethidium bromide and visualized using a UV transilluminator (Bio-Rad, USA). PCR-RFLP analysis of the 139 plasmids cloned from the 15 aforementioned GVA isolates was also performed using either *Alu* I or *Vpak11BI*, respectively (i.e. plasmid PCR-RFLP). Afterwards, the resulting products of the restriction fragments were also separated by the same system of electrophoresis.

Nucleotide sequence analysis

To avoid the cloning and sequencing of closely related variants, the plasmids from each GVA isolate that yielded different PCR-RFLP profiles were preferentially chosen for sequencing and were designated as putative variants. Primers and 3' terminal nontranslated region sequences were excluded prior to the analysis. Multiple alignments were conducted using CLUSTALX, version 1.8.1 (19). To study the inraisolate population structure, genetic variability within isolates (defined as the mean nucleotide distance between the approximately

5 plasmids obtained from each GVA isolate) was estimated using Mega 3.1 software (20). A phylogenetic tree of the 40 plasmids that were cloned from the 15 GVA isolates (plasmids in each isolate with different RFLP profiles were preferentially selected) was constructed using Mega 3.1 software, using the neighbor-joining method (21) with 1000 bootstrap replicates to assess the robustness of the nodes.

In addition, nucleotide sequence identity was assessed for sequences representing the putative phylogenetic group IV and the 3 known molecular groups (I-III), using the MegAlign feature of DNASTAR. The nucleotide sequences of 2 isolates taken from GenBank, representing each phylogenetic group, were included (group I: GTG11-1 with accession number DQ855084, and IS151 with NC003604; group II: P163-M5 with DQ855082, and GTR1SD-1 with DQ855081; group III: GTR1-1 with DQ787959, and P163-1 with DQ855088; and putative group IV: 1-19-1-2 with HQ671647, and 3-16-8-1 with HQ671660).

Variant-specific RT-PCR detection

According to the protocol (17), PCR was used to amplify the *cp* of the universal and mild GVA variants and 18S RNA of the grapevines for the 15 GVA-infected plants mentioned before, using specific primers, respectively (Table 1). RT-PCR products were visualized on a 1.5% agarose gel using a UV transilluminator (Bio-Rad).

Table 1. Oligonucleotide primers used in PCR.

Target genes	Primer pairs	Sequence (5'-3')	Amplicons (bp)
GVA (partial <i>cp</i> + full-length RNA silencing suppressor)	GVA-ORF4-1F	GGCTACGACCGAAHWATGTAC	839
	GVA-ORF5-1R	GTRTGACAACCTAGCTYGC	
Universal variants of GVA (<i>cp</i>)*	GVA6591F	GAGGTAGATATAGTAGGACCTA	271
	GVA6862R	TCGAACATAACCTGTGGCTC	
Mild variants of GVA (<i>cp</i>)*	GVA6591F	GAGGTAGATATAGTAGGACCTA	315
	GVA6906R	CCTCCTGCAGAGTTAAGGTC	
18S RNA of grapevine	Vivi-18sf	AAGCCCGATCCAGCAATA	176
	Vivi-18sr	GCCCTTTACGCCAGTCA	

The previously reported primers for the *cp* of the mild and universal variants are indicated by an asterisk (8).

Results and discussion

To investigate the incidences of viral infections in table grapes in Sichuan Province, 178 grape plants from 5 main production areas were collected, and ELISA was performed against 6 viruses. The ELISA results showed that 84 grape plants (belonging to 8 cultivars) were ELISA-negative against the 6 viruses (Table 2). Moreover, neither GFLV nor GLRaV-7 was found in any of the grape plants. The occurrence of GVA was 16.9%, and GVA was found in mixed infections with

GLRaV-3, GLRaV-2, and GVB. The large number of viral infections and the presence of mixed infections suggested that uncontrolled traffic in the propagation of infected plant material has played an important role in the spread of viruses. The relatively high mixed occurrence of GVA and GLRaV-3 in vineyards is not surprising because GVA and GLRaV-3 are both transmitted to grapevines by *Helioicoccus bohemicus* (22), and the use of own-rooted cuttings is common in the new production areas of Sichuan.

Table 2. Occurrences of single and mixed viral infections detected by ELISA.

Cultivars	No. of tested samples	GVA	GLRaV-3	GVB	GLRaV-2	GLRaV-7	GFLV
Venus	3	-	3	-	-	-	-
Red Rain	3	-	3	-	-	-	-
Jumeigui	7	-	-	-	-	-	-
Southern Resistance No.1	4	1	4	3	-	-	-
Xiahei	4	-	-	-	-	-	-
Prince Seedless	5	5	5	-	-	-	-
Himrod Seedless	15	14	14	-	-	-	-
Jingzaojing	4	-	1	-	-	-	-
Red Globe	11	-	4	1	1	-	-
Wuhezaohong	5	-	5	-	-	-	-
Tezaomeigui	4	-	1	-	-	-	-
Anni Seedless	4	-	3	-	-	-	-
Yatomi Rosa	5	-	1	-	-	-	-
Benni Fuji	5	5	5	-	-	-	-
Fujiminori	10	4	6	2	2	-	-
Baiaolin	7	-	7	-	-	-	-
Shigyoku	5	-	5	-	-	-	-
Fenghou	5	-	-	-	-	-	-
Jingya	13	-	-	-	-	-	-
Lansehaiyang	11	-	-	-	-	-	-
Kyoho	36	-	-	-	-	-	-
Hongshuangwei	4	-	-	-	-	-	-
Jingxiu	4	-	-	-	-	-	-
Centennial Seedless	4	1	1	-	-	-	-
Single incidences	178	16.9%	38.2%	3.4%	1.7%	0%	0%
Mixed infections with GVA		-	16.9%	1.7%	1.1%	-	-

The plasmid PCR-RFLP polymorphism results of 139 clones from the GVA isolates yielded different profiles, which were designated as type-I, -II, -III, -IV, and -V using *Alu* I and type-I, -II, -III, and -IV using *Vpak11BI* (Figure 1). In addition, the RT-PCR-RFLP analysis of the 15 GVA isolates showed 2 types of restriction patterns, which were designated as having either a ‘simple’ or ‘complex’ pattern. In the ‘simple’ pattern, the sum of the molecular weights of the fragments was approximately the same as that of the initial fragment (839 bp). In the ‘complex’ pattern, the total molecular weights of the individual fragments exceeded 839 bp, and the ‘complex’ pattern simultaneously consisted of more than 1 profile yielded by plasmid PCR-RFLP. In particular, grape

plant 1 showed a ‘complex’ pattern, simultaneously consisting of type-I and type-II profiles generated by plasmid PCR-RFLP for either *Alu* I or *Vpak11BI* (Figure 2). However, plant 8 had a ‘simple’ pattern containing only type-I (Figure 2). Grape plants 2, 5, 8, and 9 were characterized as having ‘simple’ patterns and showed low within-isolate genetic variability (Table 3). In contrast, other plants showed high within-isolate variability, and these GVA isolates generated ‘complex’ patterns (Table 3).

In particular, plant 8, with a ‘simple’ pattern, (only infected by type-I of the variants) showed mild stem grooving and a visibly swollen node (data not shown). By contrast, the GVA-infected plants with ‘complex’ patterns (simultaneously infected by different types of

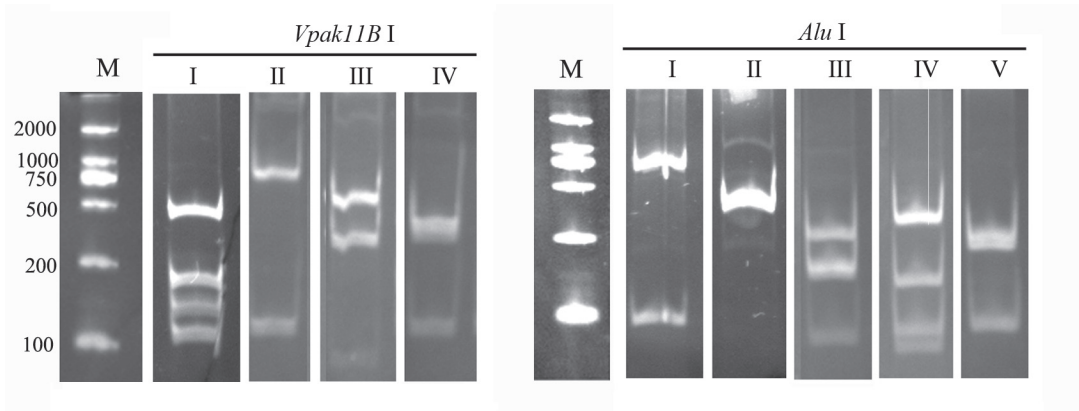


Figure 1. The profiles obtained by plasmid PCR-RFLP using *Alu* I and *Vpak11BI*.

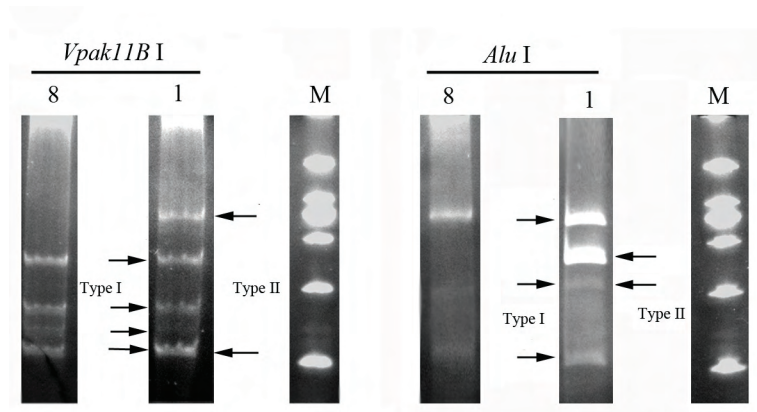


Figure 2. The ‘complex’ and ‘simple’ patterns of GVA-infected grape plants 1 and 8 were analyzed by RT-PCR-RFLP, using either *Alu* I or *Vpak11BI*, respectively. The specific bands defined as type-I and type-II profiles are indicated by arrows.

Table 3. The plasmid PCR-RFLP results of plasmids cloned from each isolate using 2 endonucleases, respectively.

Sample number	Collection location	Number of plasmids to be sequenced	Overall mean within-isolate distance \pm SD	Number of plasmids for PCR-RFLP	Alu I					V _{pk11B1}					
					I	II	III	IV	V	I	II	III	IV		
1	Ren He	6	0.121276 \pm 0.010223	6	4	2	0	0	0	0	4	2	0	0	0
2	Ping Di	5	0.008249 \pm 0.001987	7	0	0	7	0	0	0	0	7	0	0	0
3	Ren He	3	0.144288 \pm 0.11044	7	4	3	0	0	0	0	4	3	0	0	0
4	Ren He	4	0.110163 \pm 0.08523	6	1	5	0	0	0	0	1	5	0	0	0
5	Qing Long	5	0.004937 \pm 0.001693	6	0	0	6	0	0	0	0	6	0	0	0
6	Qing Long	7	0.044124 \pm 0.004681	6	0	1	5	0	0	0	0	6	0	0	0
7	Bai sha	6	0.152559 \pm 0.010245	12	8	2	2	0	0	0	8	4	0	0	0
8	Qing Long	6	0.004247 \pm 0.001632	21	21	0	0	0	0	0	21	0	0	0	0
9	Qing Long	4	0.006866 \pm 0.002163	6	6	0	0	0	0	0	6	0	0	0	0
10	Hong an	5	0.145867 \pm 0.011273	10	3	1	2	1	1	3	3	3	1	3	3
11	Hong an	5	0.109285 \pm 0.008226	11	5	1	0	3	2	2	5	1	3	2	2
12	Hong an	3	0.147527 \pm 0.011337	11	5	2	1	2	1	1	5	3	3	0	0
13	Hong an	4	0.153659 \pm 0.009750	10	3	1	2	4	0	0	3	3	4	0	0
14	Hong an	5	0.125779 \pm 0.008111	10	2	1	1	5	1	1	2	2	5	1	1
15	Hong an	5	0.108811 \pm 0.009791	10	2	1	0	1	6	1	2	1	1	6	6

variants) did not show obvious RW stem symptoms, but some leafroll symptoms were observed (data not shown). The present study revealed that table grapes (*Vitis vinifera* L. × *Vitis labrusca* L.) often carry mixed infections, characterized both by multiple viruses and by different variants of a single virus. Thus, they suffered from different RW symptoms, such as mild stem groovings or leafroll symptoms. Highly divergent GVA variant infections increased the complexity of the field symptoms. Therefore, it is difficult to specify divergent GVA variants with mild or severe pathological properties in this paper. The biological properties of different GVA variants need to be further characterized by inoculation on *Nicotiana benthamiana* or on species of *Vitis*.

Previous reports showed that the mean nucleotide diversity of the GVA *cp* was in the range of 0.104-

0.128 (9,23). In our experiment, the overall mean interisolate nucleotide distances were significantly different for the partial *cp* gene (0.161504 ± 0.017515) and the full-length RNA silencing suppressor gene (0.094098 ± 0.012698), with *cp* as more variable. However, the variability of the partial *cp* gene was consistent with the range of values (0.0020-0.224) in several structural proteins or ORFs of other plant viruses (24).

The P163-M5, GTG11-1, and P163-1 isolates from GenBank represented molecular groups I-III (8). The 40 sequences of the variants obtained from the 15 GVA isolates were deposited in GenBank under the accession numbers HQ671627 to HQ671666. In the phylogenetic tree (Figure 3), the 40 variants represented 4 phylogenetic groups and were characterized by RFLP type, respectively.

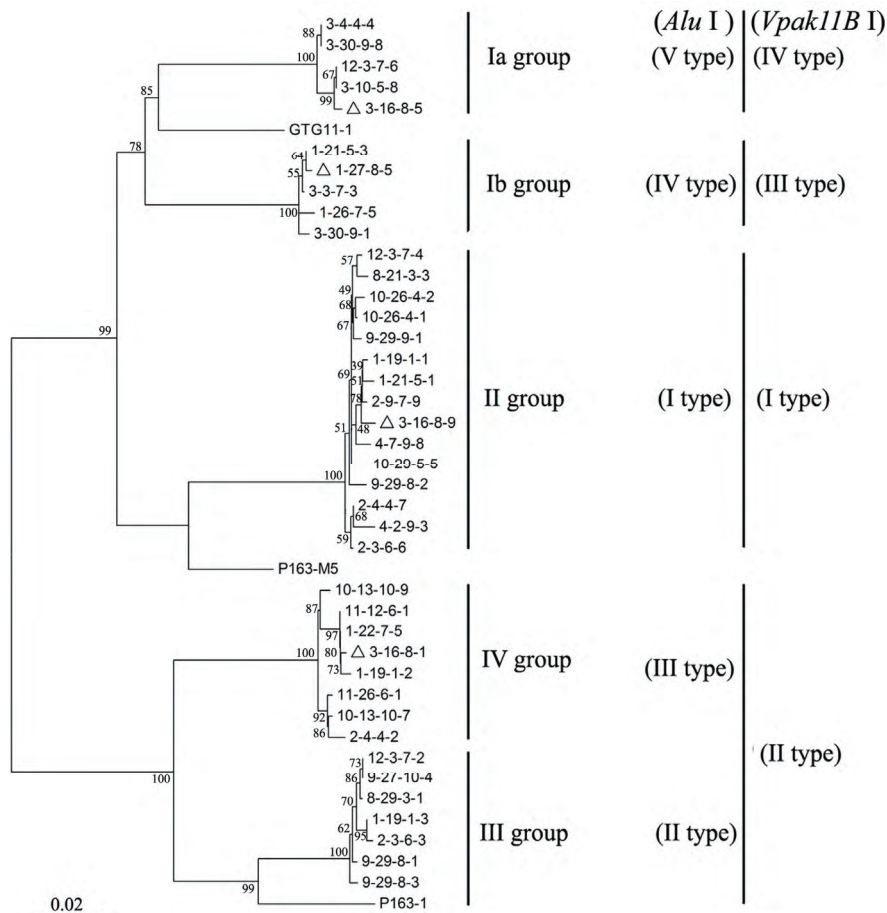


Figure 3. Phylogenetic tree of the 40 variants cloned from 15 GVA isolates. The 4 variants isolated from a GVA-infected grape plant represent different phylogenetic groups, indicated by triangular marks.

In particular, 2 subgroups (Ia and Ib) represented group I, and these subgroups were also identified by plasmid PCR-RFLP analysis (Figure 3). However, clones 3-16-8-5, 1-27-8-5, 3-16-8-9, and 3-16-8-1 were identified within a single isolate and represented group Ia, group Ib, group II, and putative group IV, respectively (Figure 3). The nucleotide identity was estimated for 8 GVA sequences from 4 groups (i.e. 2 isolates from each molecular group). Estimates ranged from 80.1% to 87.4% for the *cp* gene and 85.3% to 91.9% for the RNA silencing suppressor gene between isolates in the putative group IV and other groups (Table 4). In contrast, the nucleotide sequence identity

within putative group IV was 99% for the *cp* gene and 100% for the silencing suppressor gene (Table 4). Thus, the division of the GVA population into 4 molecular groups may be reasonable. This structure was in agreement with the result of the plasmid PCR-RFLP analysis with *Alu* I. In contrast, the plasmid PCR-RFLP analysis using *Vpak11BI* did not differentiate variants from group III and group IV.

RT-PCR was used to detect mild variants using variant-specific primers. In grape plants 2, 5, 8, and 9, RT-PCR failed to generate any detectable signals for mild variants (Figure 4). In these plants, negative results were also obtained by the more sensitive

Table 4. Nucleotide (nt) identity analysis for the GVA sequence encoding part of the *cp* gene (A) and the full-length silencing suppressor gene (B) in 8 GVA isolates clustering into 4 groups.

(A)									
Group	Isolate	Percent identity							
		GTG11-1	IS151	P163-M5	GTR1SD-1	GTR1-1	P163-1	1-19-1-2	3-16-8-1
I	GTG11-1		91.7	85.9	85.9	81.4	80.7	80.5	80.3
	IS151			87	86.5	80.1	79.9	80.3	80.1
II	P163-M5				93.2	80.1	80.1	82	81.8
	GTR1SD-1					79.1	79.1	82	82
III	GTR1-1						96.9	87.4	87.4
	P163-1							82	81.8
IV	1-19-1-2								99.2
	3-16-8-1								

(B)									
Group	Isolate	Percent identity							
		GTG11-1	IS151	P163-M5	GTR1SD-1	GTR1-1	P163-1	1-19-1-2	3-16-8-1
I	GTG11-1		91.7	85.9	85.9	81.4	80.7	80.5	80.3
	IS151			87	86.5	80.1	79.9	80.3	80.1
II	P163-M5				93.2	80.1	80.1	82	81.8
	GTR1SD-1					79.1	79.1	82	82
III	GTR1-1						96.9	87.4	87.4
	P163-1							82	81.8
IV	1-19-1-2								99.2
	3-16-8-1								

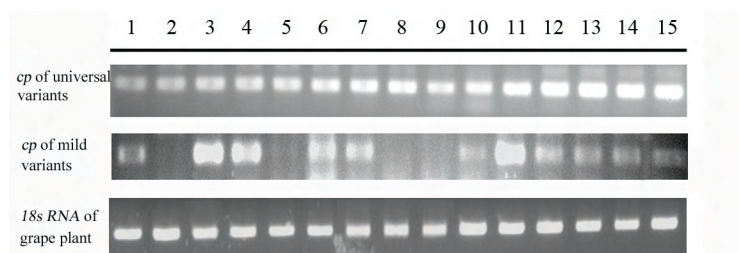


Figure 4. RT-PCR bands representing the *cp* fragments of GVA universal variants, *cp* fragments of mild variants, and 18S RNA fragments of grape plants.

quantitative RT-PCR analysis (data not shown). However, other plants were specifically infected by mild variants. Furthermore, all of the tested grape plants yielded the expected PCR products for the *cp* of GVA universal variants and grapevine 18S RNA. In addition, the same 4 GVA isolates (grape plants 2, 5, 8, and 9) produced a 'simple' pattern in the RT-PCR-RFLP analysis, and type-II profiles were not observed. However, the RT-PCR of the other 11 grape plants produced specific fragments of the mild variant and also yielded 'complex' patterns (type-II variants mixed infection with others). Thus, the type-II profile (plasmid PCR-RFLP using *Alu* I) could provide a fast and reliable indication of mild variant status for GVA-infected plants.

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