

Detection and molecular characterization of grapevine virus A in Jordan

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Summary. In a study on grapevines in Jordan conducted between 2002 and 2003, grapevine virus A (GVA) was detected in all areas where grapevines were planted. DAS-ELISA analysis of samples from symptomatic trees found that 16.1% of samples were infected with GVA. Using a GVA-specific primer pair (H587/C995), a portion of the coat protein gene of the virus was amplified by IC-RT-PCR and RT-PCR, using leaf extracts and RNA extracted from infected grapevines respectively. After cloning and sequencing the coat protein gene of the Jordanian isolate of GVA (GVA-Jo), the sequence of the amplified product was compared with sequences of other GVA isolates from different countries.

Key words: DAS-ELISA, IC-RT-PCR.

Introduction

In Jordan, grapevine (*Vitis vinifera* L.) is considered among the most important fruit crops. The amount of grapes produced in 2002 was about 34,800 tons. In the same year, the total planted area of grapevines in Jordan constituted 10.6% of the total area planted with fruit trees (Anonymous, 2002).

Several diseases caused by plant viruses are reported to infect grapevines in Jordan, among which rugose wood disease complex, grapevine fan-leaf (GFLV), grapevine leafroll (GLRV), and grapevine fleck viruses are the most important (Al-Tamimi *et al.*, 1998; Abu Shirbi, 2001)

Grapevine virus A is one of the most common viruses that cause rugose wood disease (Boscia *et al.*, 1995). In Jordan, GVA was first detected in 1975 (Savino *et al.*, 1975) and a few years later it became the most prevailing virus affecting grapevines (Boscia *et al.*, 1995; Al-Tamimi *et al.*, 1998).

GVA is distributed over long distances by propagating material and on the site by the vine mealybug *Planococcus ficus* (Minafra and Hadidi, 1994). The virus has a filamentous particle, 800 nm long that contains positive-sense single-stranded RNA of 7349 nt (Goszczynski and Jooste, 2003). The genome was completely sequenced by Minafra *et al.* (1997); it comprises five open reading frames (ORFs). ORF4 encodes the coat protein (CP), which has a single type CP subunit encoding a polypeptide of 198 amino acids with a molecular mass of 21.5–22.5 kDa (Minafra *et al.*, 1994, Minafra *et al.*, 1997).

Serological techniques such as enzyme-linked

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immuno-sorbent assay (ELISA) are the most common techniques to detect virus infections in grapevine (Rubinson *et al.*, 1997; Al-Tamimi *et al.*, 1998; Acheche *et al.*, 1999). In addition, several molecular techniques, such as polymerase chain reaction (PCR) have been widely used for the diagnosis of plant viruses, allowing the detection of very small amounts of a virus, and also the cloning of genomic fragments of viruses (Henson and French, 1993).

Reverse transcription (RT)-PCR, and immunocapture (IC)-RT-PCR are two PCR techniques that have been previously described to diagnose grapevine diseases caused by plant viruses (Minafra *et al.*, 1992; Nassuth *et al.*, 2000; M'hirsi *et al.*, 2001).

The aims of this study were to study the spread of GVA in grapevine growing areas in Jordan and to identify GVA at the molecular level.

Materials and methods

Sample collection

Leaf samples from grapevines with rugose wood disease symptoms were collected from different locations. Diseased vines were dwarfed, showed delayed bud opening in spring, and swelling above the bud union. A total of 81 leaf samples were collected from the research station of Al-Balqa' Applied University and from the Ajloun, Al-Salt, Ramtha, and Al-Mafraq areas (Table 1). Samples were placed in plastic bags and kept at 4°C for further use.

Double-antibody Sandwich (DAS)-ELISA

DAS-ELISA was performed following the general protocol of Clark and Adams (1975). The poly-

clonal antibodies against GVA developed by Sediag (Sediag S.A., Strasbourg, France) were used according to manufacturer's instructions. Leaf samples were prepared for DAS-ELISA by peeling 0.5 g of the midribs from the leaves of young flush, chopping finely with a razor blade, and homogenizing in 5 ml of grape extraction buffer (0.5 M Tris-base, 0.14 M sodium chloride, 0.5 mM polyvinylpyrrolidone [PVP], 2 mM polyethylene glycol, 3 mM sodium azide, and 0.05% Tween-20; pH 8.2). Extracts were clarified by centrifugation at 14,000 g for 5 min and supernatants were kept on ice. Wells of microtiter plates (Nalge Nuc International, New York, NY, USA) were coated with 100 µl of GVA polyclonal antibodies diluted to 1:1000 in 50 mM sodium carbonate buffer (pH 9.6) and incubated at 37°C for 4 h. Plates were washed 3 times with phosphate buffer saline, pH 7.2–7.4, supplemented with 0.1% Tween 20 (PBST). After that, 100 µl of plant extracts was loaded into each well and plates were incubated at 4°C for 16 h. After washing 3 times with PBST, 100 µl of an appropriate dilution of the conjugated antibodies was applied and plates were incubated at 37°C for 2 h. Plates were washed extensively with PBST, and 100 µl of alkaline phosphatase solution (p-nitrophenyl phosphate, 1 mg ml⁻¹ in substrate buffer) (Sigma, New York, NY, USA) was added to each well. Plates were incubated at room temperature and read at 405 nm after 90 min using a Dynex MRX ELISA plate reader (DYNEX Technologies Inc, Dinex, Chantilly, VA, USA). Each sample was analyzed in at least two wells, and the mean experimental readings that were at least three times the mean reading of the negative controls were considered positive. Grape-

Table 1. Detection of grapevine virus A in symptomatic grapevines samples from different locations in Jordan.

Location	Symptomatic samples	Diagnostic technique		
		DAS-ELISA	IC-RT-PCR ^a	RT-PCR
Al-Balqa' Applied University	41	0	-	-
Ajlun	17	2	2	1
Al-Salt	7	7	3	1
Ramtha	11	0	-	-
Al-Mafraq	5	4	1	0
Total	81	13	6	2
% of infection		16.1	7.4	2.4

^a Only ELISA-positive samples were tested with RT-PCR.

vine samples that showed high ELISA values were selected for IC-RT-PCR and RT-PCR analysis.

IC-RT-PCR

Samples to be used for IC-RT-PCR were extracted as indicated previously for the DAS-ELISA test. Sterile polypropylene PCR tubes were precoated with 200 μ l of Agritest anti-GVA-IgG (1:1000 w:v in 50 mM sodium carbonate buffer, pH 9.6). Tubes were incubated at 37°C for 4 h and then rinsed three times with PBST. To each tube 200 μ l of plant extracts from GVA-infected or healthy grapevines was added and tubes were incubated at 4°C for 16 h. After washing with PBST, tubes were incubated at 65°C for 10 min and then kept on ice.

PCR was performed using the Access RT-PCR System (Promega Corp., Madison, WI, USA) according to manufacturer's instructions. To amplify the CP gene of GVA, the primer pair H587 (5'-GACAAATGGCACACTACG-3') and C995 (5'-AAGCCTGACCTAGTCATCTTGG-3'), previously described by Minafra and Hadidi (1994), was used. In a final volume of 50 μ l, a PCR mixture consisting of: 10 μ l of 10 \times AMV/*Tfi* PCR buffer, 1 μ l of 10 mM dNTP mixture, 2 μ l of 25 mM MgSO₄, 1 μ M of each primer, 25 μ l of sterile distilled water, 1 μ l of *Tfi* DNA Polymerase (5 U μ l⁻¹) and 1 μ l of AMV Reverse Transcriptase (5 U μ l⁻¹), was added to PCR tubes precoated with GVA IgG. To facilitate cDNA production, tubes were incubated at 48°C for 45 min and PCR was performed in a programmable thermal controller (model PTC-200, MJ Research Inc., Watertown, MA, USA). The following parameters were used: one cycle at 94°C for 5 min; 5 cycles at 94°C for 50 sec, 50°C for 1 min, and 72°C for 1 min; followed by 30 cycles at 94°C for 50 sec, 51°C for 1 min, and 72°C for 1 min. After a final extension at 72°C for 10 min, PCR products were analyzed by agarose gel electrophoresis.

RNA extraction

Two methods were followed to extract total RNA from grapevine tissues. The first method was based on a modified protocol described by Nassuth *et al.* (2000). RNA was extracted using the components of the Plant RNeasy kit (Qiagen, Valencia, CA, USA). Leaf petioles were homogenized in lysis buffer (4 M guanidium thiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 6% PVP and 1% 2-mercaptoethanol) (1:10 w:v). One milliliter of the lysate was

mixed with 67 μ l of 30% Sarkosine and incubated for 10 min at 70°C. The mixture was applied to a Qiashreder spin column (Qiagen) and centrifuged at 20,000 rpm for 2 min. After that, 250 μ l of the mixture was transferred to a new tube, mixed with 225 μ l of 95% ethanol and applied to a RNeasy column (Qiagen). The column was centrifuged at 10,000 rpm for 45 sec and washed. Total RNA extracts (100 μ l) were stored in small aliquots at -80°C (stock sample) and at -20°C (working sample).

The second extraction method followed the protocol of Gauthier *et al.* (1997) with minor modifications. Total RNA was extracted from grapevine petioles. In brief, 100 mg of leaf petioles was homogenized in 1 ml GTC solution (4 M guanidium thiocyanate, 25 mM sodium acetate pH 7, 0.5% SDS and 0.1 M 2-mercaptoethanol) and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to another tube and 100 μ l of sodium acetate (2 M) and 1 ml of chloroform:isoamyl alcohol (42:1) was added. Tubes were placed on ice for 15 min and then centrifuged at 14,000 rpm for 15 min. The RNA was precipitated with 95% ethanol, washed with 75% ethanol and resuspended in 100 μ l RNase-free water. Aliquots of RNA were stored at -20 or -80°C.

RT-PCR

RT-PCR was carried out on RNA extracted from GVA-infected and from healthy grapevine tissues according to Nassuth *et al.* (2000). The RT-PCR mixture contained 2.5 μ l RNA, 10 mM MgCl₂, 0.2 mM dNTPs, 1 mM MgSO₄, 10 mM DTT, 2% sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 μ l of each primer, 1 \times *Taq* buffer, 1.25 μ l of 2 U μ l⁻¹ AMV enzyme, and 2 μ l *Taq* enzyme. The reverse transcription reaction was carried out at 42°C for 60 min, and then PCR was performed using the same parameters as described above for IC-RT-PCR. PCR products were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis

Aliquots (5 μ l) of PCR products were analyzed on a 1% agarose gel in 0.5 \times TBE buffer (90 mM Tris-borate, 2 mM EDTA) followed by staining with ethidium bromide (0.5 μ g ml⁻¹). DNA fragments were visualized with a UV transilluminator and photographed with the Gel Doc 2000 (BIO-RAD, Hercules, CA, USA). DNA molecular weight mark-

ers (Promega) were used to determine the size of the amplified fragments.

cDNA cloning and sequencing of the CP gene

The PCR product was ligated to the pGEM®-T Easy Vector (Promega) and cloned according to manufacturer's instructions. Colonies containing recombinant plasmids were selected and controlled by restriction digestion. One clone of the GVA isolate was taken for sequencing. Both orientations of the clone were sequenced by an automatic sequencer at the Biotechnology Center, Madison, WI, USA, using the Big Dye sequencing kit (Applied Biosystems, Foster City, CA, USA). Alignment analysis was carried out with the DNAMAN software program (Lynnon BioSoft, Que., Canada).

Results

DAS-ELISA

The DAS-ELISA technique detected GVA in 13 of the 81 samples (Table 1). All samples from the Al-Salt area were infected with GVA. However, none of the samples from the research station of the Al-Balqa' Applied University or from the Ramtha areas reacted positively with the IgG of GVA. Only two samples from Ajlun were infected with GVA. According to the DAS-ELISA results, 16.1% of all samples were infected with GVA.

Amplification of the CP gene with IC-RT-PCR and RT-PCR

To confirm the results of the DAS-ELISA test, samples that were positive with GVA-IgG were analyzed with IC-RT-PCR and RT-PCR. The expected size (430 bp) of the CP gene was amplified by IC-RT-PCR using the primer pair H587/C995 (Fig. 1). Figure 2 shows the amplification of the CP gene of GVA by RT-PCR from symptomatic grapevine samples. The expected size of the CP gene was amplified in RNA extracted with the method of Nasuth *et al.* (2000) (Fig. 2), but was not amplified in RNA extracted using the method of Gauthier *et al.* (1997). No bands were detected in extracts from healthy grapevine trees. The identity of the amplicon was confirmed by cloning and sequencing.

cDNA cloning and sequencing of the CP gene

The amplified fragment of GVA CP was ligated and cloned successfully into the pGEM®-T Easy

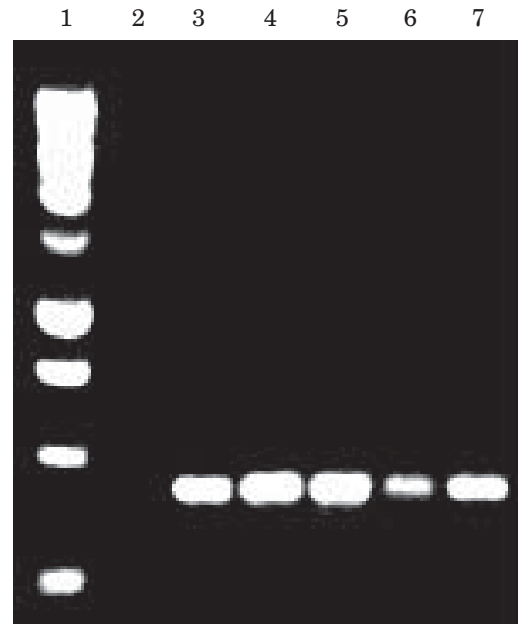


Fig. 1. Agarose gel electrophoretic analysis of the coat protein gene of grapevine virus A amplified by IC-RT-PCR using the primer pair H587/C995. Aliquots (5 μ l) of the PCR products were analyzed in a 1% agarose gel. Lane 1, 1-Kb DNA size marker; lane 2, negative control; lanes 3–7, symptomatic field samples.

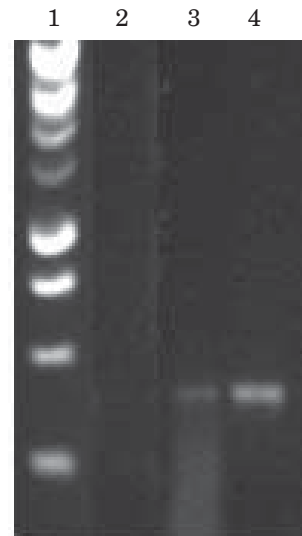


Fig. 2. Agarose gel electrophoretic analysis of the coat protein gene of grapevine virus A amplified by RT-PCR using the primer pair H587/C995. Aliquots (5 μ l) of the PCR products were analyzed in a 1% agarose gel. Lane 1, 1-Kb DNA size marker; lane 2, negative control; lanes 3 and 4, symptomatic field samples.

Table 2. Nucleotide sequence homology of the coat protein gene of the Jordanian isolate of grapevine virus A with isolates from other countries.

GVA isolate ^a	Accession No.	Origin	Coat protein homology (%)
92/778	AF441234.1	South Africa	90
Is 151	X75433.1	Italy	89
-	AF494187.1	Brazil	88
PA3	AF007415.2	Israel	86
P163-1	AF441236	South Africa	83
JP98	AF441235.1	South Africa	83

^a GVA isolates available in the GenBank and used in this study for comparison with the Jordanian isolate of GVA.

vector. Sequencing results (Fig. 3) confirmed that the PCR products obtained with the primer pair H587/C995 were indeed the CP gene of GVA. The sequence of the CP gene of the Jordanian isolate (GVA-Jo) was deposited in the GenBank under accession No. AY594176. This sequence was homologous to sequences of the CP gene of other GVA isolates in the GenBank (Fig. 3). Alignment analysis of the GVA sequence revealed 90 and 89% identity with GVA-92/778 and GVA-Is-151 from South Africa and Italy respectively (Table 2). On the other hand, the identity between GVA-Jo and GVA isolates PA3 and P163-1 was 86, 83 and 83% respectively.

Discussion

Although all samples showed rugose wood symptoms, only 16.1% of samples were infected with GVA. This low percentage might be due to the fact that rugose wood is a complex disease that can be caused by different viruses such as grapevine fleck virus, grapevine leafroll-associated closterovirus 1 and 3, as well as by GVA and the symptoms observed may have been caused by one or more of those other viruses (Credi, 1997; M'hirsi *et al.*, 2001). Another explanation is that the concentration of GVA in symptomatic samples was too low to be detected by ELISA. In another study, Al-Tamimi *et al.* (1998) reported a 47.5% incidence of GVA in samples from locations other than those investigated in this study.

DAS-ELISA is a common diagnostic technique in survey studies to detect plant viruses. However,

the technique is reported to have some disadvantages. These include an inconsistency of results in detecting plant viruses in fruit trees, and a low efficiency in detecting viruses at low concentrations (MacKenzie *et al.*, 1997). Molecular techniques such as PCR and nucleic acid hybridization have however been successful in detecting virus and virus-like diseases in fruit trees (Saldarelli *et al.*, 1993; Habili *et al.*, 1995; Kummert *et al.*, 2001). Therefore a nation-wide survey should be done in Jordan to study the natural spread of GVA in grapevine-growing areas using molecular techniques such as nucleic acid hybridization.

IC-RT-PCR, which is based on the concentration and immobilization of the target virus with polyclonal or monoclonal antibodies (Fattouch *et al.*, 2001), was used in this study to amplify part of the CP gene of GVA. Although samples used for the IC-RT-PCR reacted positively with the IgG of GVA in the DAS-ELISA test, the expected size of the CP gene was amplified in only 6 samples (Table 1). A possible reason for this is that some samples reacted nonspecifically with the antibodies raised against GVA. In addition, samples that were not detected with the IgG of GVA may have been infected with another virus that also causes rugose wood disease symptoms. These results are consistent with previous studies that showed that IC-RT-PCR is more sensitive than ELISA in detecting plant viruses (Henson and French, 1993; Minafra and Hadidi, 1994; Acheche *et al.*, 1999; Fattouch *et al.*, 2001; M'hirsi *et al.*, 2001).

With RT-PCR analysis, the CP gene of GVA was detected in only 2 samples from which RNA was extracted according to Nassuth *et al.* (2000), but not from any samples from which RNA was extracted with the method of Gauthier *et al.* (1997). The difference between the two RNA extraction protocols is that the extraction buffer of Nassuth *et al.* (2000) contains PVP, which binds phenolic compounds, while the extraction buffer of Gauthier *et al.* (1997) does not. Grapevines, like other woody plants, contain high amounts of phenolic compounds and polysaccharides which can interfere with RT-PCR (Demeke and Adams, 1992; John, 1992), and since the extraction buffer of Gauthier *et al.* (1997) did not include PVP it may have prevented the synthesis of cDNA and hence the amplification of the CP gene. In addition, the method of Nassuth *et al.* (2000) uses the Plant RNeasy kit

GVA-Jo	<u>GACAAATGGCACACTACG</u> CAAAGAGGGTGGAGATACGCGC	40
GVA-Is 151	-----c-----a-----a-g--	40
GVA-Brazil	-----c-----c-----a-g--	40
GVA-92/778	-----t--t-----c-----a-g--	40
GVA-Jo	GATAATAGAGGAGTTGGTTCTGGCGAAAGCCCAACCAACC	80
GVA-Is 151	-----a-----gt-----t	80
GVA-Brazil	-----a-----g-----g-t	80
GVA-92/778	-----a-----g-----g-----t	80
GVA-Jo	GATGACGCTTCCGAGAGCGGCTACGACCGAACTATGTACC	120
GVA-Is 151	--c-----t-----a-----	120
GVA-Brazil	--a----c--t-----a-----	120
GVA-92/778	-----	120
GVA-Jo	TAAATACTCTCTTTGGGTACATCGCCTTGGTCGGGACAAG	160
GVA-Is 151	-g--c-----c-----	160
GVA-Brazil	-g-----c-----t----	160
GVA-92/778	-g-----c-----t----	160
GVA-Jo	CAAAAAGGCGGTCCATTATGGAGAGGTAGATATAGTAGGT	200
GVA-Is 151	-----a-t-----g-----	200
GVA-Brazil	-----c-----g-----	200
GVA-92/778	-----c-----g-----	200
GVA-Jo	CCTAAAGCTAGCAAAAAGACGGGGATCGATCCAAGGGGAA	240
GVA-Is 151	-----g-----c--c-----g-	240
GVA-Brazil	-----a--a--c-----	240
GVA-92/778	-----a--a--a-----g-----	240
GVA-Jo	AGATTGTCATATCAGAACTGGTCGGCAGGATGCGCACTCT	280
GVA-Is 151	---g--gg-g--g--g-----g--	280
GVA-Brazil	--t-g--g-g-----g-----g--	280
GVA-92/778	--t-g--g-g-----g-----	280
GVA-Jo	AAGTGTGGCCGTGAGCGAGGGCCCTGTTAAGGGGGCAACA	320
GVA-Is 151	g-----a--a-----a--c--c-----t	320
GVA-Brazil	g----t--a-----a--a--c-----c	320
GVA-92/778	g--c-----a--c-----a--c--c-----c	320
GVA-Jo	TTGAGGCAGATGTGCGAGCCATTGCCCCAGAATGCCTACG	360
GVA-Is 151	c-----a-----c-----	360
GVA-Brazil	c-----a--t--a--a--c-----t-	360
GVA-92/778	c-a-----a-----a-----c--t--	360
GVA-Jo	ATTTCCCTCGTGTTGATGGCTGAAATGGGCACGTATTACACA	400
GVA-Is 151	-----ag-----a--c-----	400
GVA-Brazil	-c-----cc-----g-----a-----	400
GVA-92/778	-----ac-----g-----c-----	400
GVA-Jo	GTTGGCTA <u>CCAAGATGACTAGGTCAGGCTT</u>	430
GVA-Is 151	---a--c-----	430
GVA-Brazil	---a--a-----c-----	430
GVA-92/778	---a-----	430

Fig. 3. Multiple alignment of coat protein gene sequences of grapevine virus A from Jordan (GVA-Jo) (GenBank accession No. AY594176), from Italy (GVA Is 151) (GenBank accession No. X75433.1), from Brazil (GenBank accession No. AF494187.1) and from South Africa (GVA 92/778) (GenBank accession No. AF441234.1). Dashed line indicates an identical base at a given position. Sequences of H587 and C995 primers are underlined

for RNA extraction. The RNA obtained is expected to be of good quality and to contain less inhibitor. These RT-PCR results were consistent with previous studies that showed the importance of PVP in extracting RNA for the diagnosis of plant viruses using the RT-PCR technique (Acheche *et al.*, 1999; Salzman *et al.*, 1999; Nassuth *et al.*, 2000).

To our knowledge, this is the first study in Jordan that characterizes GVA at the molecular level. Previous studies reported the occurrence of the virus (Boscia *et al.*, 1995; Al-Tamimi *et al.*, 1998) but did not provide information about it at the molecular level. Sequencing results of a portion of ORF 4 (Fig. 4) confirmed the IC-RT-PCR and RT-PCR analysis and showed slight variations between the sequence of the CP gene of GVA-Jo and that of other strains from other parts in the world.

The study provides clear evidence that GVA occurs in Jordan. However, from an epidemiological point of view; much work still needs to be done. For example, the source of GVA must be investigated and its spread to locations where it does not occur should be prevented. Here, it is important to note that, due to the lack of effective certification of fruit trees against virus diseases in Jordan, budwood material is usually imported from abroad without being inspected at the border for virus and virus-like diseases. It is necessary to put an end to such uncontrolled imports.

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