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Molecular characterization of *Grapevine virus A* isolates from Jordan

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Summary. A total of 1141 samples of petioles and canes of grapevines from different locations in Jordan were tested by DAS-ELISA for *Grapevine virus A* (GVA). About 14.2% of samples were infected with GVA. Using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) or Immunocapture (IC)-RT-PCR, a fragment of 430 bp of the coat protein gene was amplified using the primer pair H587/C995. Fifteen clones were sequenced and aligned with each other and with the Jordanian isolate (GVA-Jo) using the DNAMAN program. Alignment analysis showed that all Jordanian isolates shared 100% nucleotide identity with each other and with the Jordanian isolate GVA-Jo. To classify the collected GVA isolates in one of the GVA groups a pairwise nucleotide alignment between isolate GVA-5R and isolates from South Africa; 92/778, JP98 and P163-1 representing GVA group I, II and III respectively was done. Alignment results indicated that isolate GVA-5R shared 90, 83, and 76% nucleotide similarity with GVA groups I, II, and III, respectively.

Keywords: Vitivirus, DAS-ELISA, RT-PCR, DNA sequencing.

Introduction

Grapevine virus A (GVA) is the type species of the genus *Vitivirus* in the family *Flexiviridae* (Martelli *et al*., 1997; Adams *et al.,* 2004). GVA has a filamentous flexuous particle, and is 800 nm in length, and 11–12 nm in diameter (Conti *et al.,* 1980). The positive-sense-single stranded RNA (+ssRNA) genome is organized into five Open Reading Frames (ORFs1-5). ORF1 encodes replication proteins (194 KDa) (Minafra *et al*., 1997). ORF2, which is partially overlapped by ORF1 and ORF3, encodes a polypeptide of 19 KDa that has no homology to any known proteins but has some similarity to the polypeptide encoded by ORF2 of GVB (Saldarelli *et al*., 1996). ORF3 encodes a putative movement protein (31 KDa), while ORF4 encodes the coat protein (21.5 KDa), and ORF5 (10 KDa) encodes a suppressor of RNA silencing and has a role in pathogenicity (Minafra *et al*., 1994; Galiakparov, 2003). Biologically, GVA isolates from South Africa were separated according to the symptoms they induced on mechanically inoculated *N. benthamiana* (Goszczynski and Jooste, 2002 and 2003 a,b). These isolates induced four kinds of symptoms: 1, mild vein clearing; 2, vein clearing with interveinal chlorosis; 3, vein clearing, interveinal chlorosis and strong curling of top leaves; and 4, extensive patchy necrosis. On the other hand, molecular analysis of the nucleotide sequence of the 3' region revealed that GVA isolates can be divided into 3 groups (I, II, and III), sharing 91.0–

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99.8% nt sequence identity within groups and 78.0–89.3% nt sequence identity among groups. Mild isolates are classed in group III and share only 78.0–79.6% nt sequence with other isolates (Goszczynski and Jooste, 2003a).

The study of variability is one of the most important aspects of plant virology because strains vary in the severity of the disease they cause in the field, and this variation may be highly relevant to the development of control strategies of viral diseases. Variability is also important for understanding how viruses have evolved and are evolving (Arenal *et al.*, 2001). Although an isolate from Jordan (GVA-Jo) was characterized by Anfoka *et al*. (2004), who found that the coat protein DNA fragment of 430 bp had an 83–90% nucleotide identity with various isolates (92/778, JP98, and P163-1) of GVA, information about the molecular characterization of different Jordanian isolates of GVA is still lacking.

Materials and methods

Sample collection and GVA detection by the double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)

Leaf petioles and cane samples from symptomless grapevines as well as from grapevines with leafroll symptoms were tested for GVA by DAS-ELISA as described by Clark and Adams (1977), using the GVA ELISA kit developed by Bioreba AG (Reinach, Switzerland) according to the manufacturer's instructions. Absorbance was measured at 405 nm (A_{405}) after 2 h using the ELISA microplate reader (Expert Plus, Hitech, Eugendorf, Austria). Each sample was analyzed in two wells, and mean readings at least two times the mean reading of the negative controls were considered positive.

RNA extraction

Total RNA was extracted from infected grapevine samples as described by Sambrook and Russell (2001) with minor modifications. One tenth of a gram of cane scrapes or leaf petioles was ground in liquid nitrogen. The powder was transferred to a 2-ml sterile Eppendorf tube and 1 ml of a guanidinium thiocyanate (GTC) solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 5 g l^{-1} N-lauryl sarcosine, 0.1 M 2-mercaptoethanol), 800 μl of 1 M phenol (pH 4), 150 μl of 3 M sodium acetate (pH 4.0) and 200 μl chloroform:isoamyl alcohol (24:1) were added. The homogenate was vortexed vigorously for 10 s and kept on ice for 15 min. Tubes were centrifuged at 20,000 rcf for 10 min. at 4ºC using a microcentrifuge (Eppendorf, 5417R, Hamburg, Germany). The upper aqueous phase was transferred to a new tube containing 150 μl sodium acetate and 800 μl chloroform:isoamylalcohol (24:1) and centrifuged at 20,000 rcf for 10 min. The supernatants were added to a 2/3 volume of cold isopropanol and incubated at -20ºC for 1 h. Pellets were collected by centrifugation at 20,000 rcf for 15 min., were washed twice with 70% ethanol, air dried, redissolved in 60–80 μl of 1 ml l^{-1} DEPC–treated water, and stored at -20 or -80ºC for further use.

Reverse transcription-polymerase chain reaction (RT-PCR)

The primer pair H587 (5´-GACAAATGGCACACTACG-3´)/C995 (5´-AAGCCTGACCTAGTCATCTTGG-3´), designed by Minafra *et al*. (1992), was used to amplify a 430 bp fragment of the GVA coat protein (CP) gene. RT-PCR was performed using the Access RT-PCR System (Promega Corp., Madison, WI, USA) according to manufacturer's instructions. The reaction was carried out in a final volume of 25 μl that consisted of: 2.5 μl RNA, 5 μl of 5× AMV/*Tfl* reaction buffer, 1 μl of 25 mM $MgSO_4$, 1 μl of dNTPs mix (10 mM of each dNTP), 0.5 μl AMV reverse transcriptase (5 U μl^{-1}), 0.5 μl of *Tfl* DNA polymerase (5 U μl^{-1}), 1.5 μl of each primer (20 μM), and 11.5 μl nuclease free water. To facilitate cDNA synthesis, 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 2 min; followed by 30 cycles at 94°C for 50 s, 55°C for 50 s, and 72°C for 1 min. After a final extension step at 72°C for 10 min. the PCR tubes were held at 4°C or stored at -20°C.

Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

Samples to be used for IC-RT-PCR were extracted as indicated for DAS-ELISA. To each tube, 200 μl of plant extracts from GVA-infected grapevines was added and tubes were incubated at 4°C for 16 h. After washing with phosphate buffer saline-tween (PBS-T), tubes were incubated at 65ºC for 6

min and then kept on ice. PCR was performed using the primer pair H587/C995. The PCR mixture was prepared as above except that the RNA was replaced by nuclease free-water. Tubes were incubated at 48°C for 45 min. The following PCR parameters were used: one cycle at 94°C for 5 min, 5 cycles of 94ºC for 50 s, 50ºC for 1 min, and 72ºC for 1 min, then 30 cycles of 94ºC for 50 s, 51°C for 1 min and 72ºC for 1 min followed by a final extension step at 72ºC for 10 min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing 0.5 μg ml^{-1} ethidium bromide (EtBr). DNA fragments were visualized using a UV transilluminator (FLUO-LINK, FLX, Vilber Lourmat, Torcy, France) and the size of the PCR products was determined using a 100 bp DNA ladder (Promega). Photographs of gels were done using a computer imaging system equipped with a digital camera (Kodak, EDAS 290).

Cloning and sequencing

PCR products were cloned using the pGEM®-T Easy Vector System II (Promega). The ligation reaction and transformation using *Eschericha coli* were carried out according to manufacture's instructions, while recombinant plasmids were isolated using the method described by Sambrook and Russell (2001), and then monitored for the appropriate-size inserts (430 bp) by cleavages with *Eco*RI restriction enzyme. To confirm the identity of the amplified fragments, plasmids containing cDNAs of the predicted size (430 bp) were used to determine the nucleotide sequence of the amplified product. Fifteen clones bearing recombinant plasmids containing the gene of inserts were sent to Macrogen (Seoul, South Korea) for sequencing. Alignment analysis between the sequences of different Jordanian GVA isolates was carried out using the DNAMAN software program (Lynnon BioSoft, Quebec, Canada). The DNA sequence and amino acid sequences of one GVA isolate (GVA-5R) was compared with sequences of DNA and amino acids of other GVA isolates reported from different parts of the world available at the GenBank site www.ncbi.com. These isolates were GVA-Jo (AY594176), 92/778 (AF441234.1), JP98 (AF441235.1) and P163-1 (AF441236.1). The last three isolates represent the three GVA groups (I, II, and III) described by Goszczynski and Jooste (2003 a, b).

Results

Detection of GVA

GVA detection was carried out by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA), reverse transcription–polymerase chain reaction (RT-PCR) and immunocapture (IC)-RT-PCR

About 14.2% of the 1141 grapevine sample tested positively for GVA (data not shown). To amplify the CP gene of GVA from the collected samples, 61 grapevine samples that showed a positive reaction with DAS-ELISA were subjected to RT-PCR or IC-RT-PCR. Using specific primer pairs, the expected size of the CP gene fragment (430 bp) of GVA (Fig. 1) was amplified from 30 of the 61 samples.

Cloning and sequencing of the coat protein gene fragment.

The amplified fragment of GVA CP was ligated and cloned successfully into the pGEM®-T Easy vector. Transformed bacterial white colonies were selected and the presence of the insert 430 bp fragment in the plasmid vector was confirmed by restriction digestion with *Eco*R1. The nucleotide sequences of the fifteen Jordanian isolates were compared and indicated that all isolates had nu-

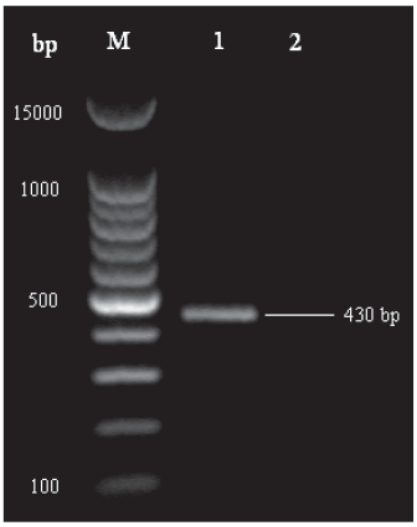

Fig. 1. Agarose gel electrophoretic analysis of the coat protein gene fragment of *Grapevine virus A* amplified by RT-PCR using the primer pair H587/C995. Aliquots (10 μl) of the PCR products were analyzed in a 1.5% agarose gel. M, 100 bp DNA ladder; lane 1, field sample; and lane 2, negative control.

cleotide sequence identity (100%) (data not shown). Alignment analysis of the GVA sequences revealed 100% identity with the GVA-Jo isolate previously described by Anfoka *et al*. (2004). A comparison of nucleotides and amino acid sequences between GVA-5R and other isolates

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GVA-5R.SEQ GACAAATGGCACACTACGCAAAGAGGGTGGAGATACGCGC     40
92-778.SEQ ----------t--t-----c---------------a-g--     40
JP98.SEQ   ----------t----g--------a------a--ga-g--     40
P136-1.SEQ ---tc----gt--g-t-----g-----g------c--a--     40

GVA-5R.SEQ GATAATAGAGGAGTTGGTTCTGGCGAAAGCCCAACCAACC     80
92-778.SEQ ---------a--------g--------------g-----t     80
JP98.SEQ   t--c--c----------g------------acc-----t     80
P136-1.SEQ -g-g--------ac-c--g--c----g---gg-g-tg-gt     80

GVA-5R.SEQ GATGACGCTTCCGAGAGCGGCTACGACCGAACTATGTACC    120
92-778.SEQ ------------------------------------------  120
JP98.SEQ   --a------a-t--------------------a-------   120
P136-1.SEQ --g---a-gag-acag-a--------------a-------   120

GVA-5R.SEQ TAAATACTCTCTTTGGGTACATCGCCTTGGTCGGGACAAG    160
92-778.SEQ -g----------c--------------------t-----   160
JP98.SEQ   -g----------c--------------------t--t--   160
P136-1.SEQ -g----------c--------------------t--t--   160

GVA-5R.SEQ CAAAAAGGCGGTCCATTATGGAGAGGTAGATATAGTAGGT    200
92/778.SEQ ----------------c-----g-----------------   200
JP98.SEQ   ----------a--------------------------a    200
P136-1.SEQ ---g------act--------------------------a   200

GVA-5R.SEQ CCTAAAGCTAGCAAAAAGACGGGGATCGATCCAAGGGGAA    240
92/778.SEQ --------------------a--a--a-----g-------   240
JP98.SEQ   ----g---------g----t------a--c--gc-t--g-   240
P136-1.SEQ -----t--a--t--g-----t--cc-a--c--t-----t-   240

GVA-5R.SEQ AGATTGTCATATCAGAACTGGTCGGCAGGATGCGCACTCT    280
92/778.SEQ --t-g---g-g----------g-----------------   280
JP98.SEQ   --t-g--tg-g--c--g-----g--a------a-g-----   280
P136-1.SEQ -a--aaa-g-gg-tagca----g-cgc-c---a-a-----   280

GVA-5R.SEQ AAGTGTGGCCGTGAGCGAGGGCCCTGTTAAGGGGGCAACA    320
92/778.SEQ g--c-----a--c--------a--c--c-----------c   320
JP98.SEQ   g--c-----t--a--------g--c--c--a-----t---   320
P136-1.SEQ c----c---t--a--------g-----c-----------t   320

GVA-5R.SEQ TTGAGGCAGATGTGCGAGCCATTCGCCCAGAATGCCTACG    360
92/778.SEQ c-a--------------a--------a-----c--t----   360
JP98.SEQ   c-c----------t--a--t-----a-----c-----t-   360
P136-1.SEQ c----------------a--g--t-----a--c--t----   360

GVA-5R.SEQ ATTTCCTCGTGTTGATGGCTGAAATGGGCACGTATTCACA    400
92/778.SEQ ----------ac----------g-----------c-----   400
JP98.SEQ   -------------a-----g-----------t--c--g--   400
P136-1.SEQ -------g--cg-t--------gt--a-t------a-g--   400

GVA-5R.SEQ GTTGGCTACCAAGATGACTAGGTCAGGCTT             430
92/778.SEQ ---a--------------------------             430
JP98.SEQ   ---a--a--t--------a-----------             430
P136-1.SEQ ---a--a----------------g-----              430
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Fig. 2. Nucleotide sequence alignment of the CP gene of GVA between the Jordanian isolate GVA-5R and isolates 92/778, JP98, and P163-1. Dashed line (-) indicates identical base at a given position. Differences in nucleotide sequences are indicated in small letters.

Table 1. Comparison of sequence similarities of the amplified coat protein (CP) gene fragment (430 bp) of *Grapevine virus A* isolate GVA-5R and other GVA isolates from South Africa.

GVA isolate[a]	Accession No.	GVA group	Nucleotide identity (%)	Amino acid similarity (%)
92/778	AF441234.1	I	90.2	98
JP98[2]	AF441235.1	II	83.5	92
P163-1[3]	AF441236.1	III	76.3	80

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

showed that the GVA-5R isolate (one of the present isolates) shared 90, 83 and 76% nucleotide identities (Fig. 2) and 98, 92 and 80% amino acid similarities with isolates 92/778 (AF441234.1), JP98 (AF441235.1) and P163-1 (AF441236.1) respectively (Table 1).

Discussion

Grapevine virus A occurred in most of the areas surveyed. These results are in accordance with previous studies (Boscia *et al.,* 1995; Al-Tamimi *et al.,* 1998; Anfoka *et al*., 2004). Recent advances in molecular biology have led to the development of new techniques that overcome the limitations of ELISA. Previous studies showed that the CP gene of GVA had a certain degree of variability (DeMeyer *et al*., 2000; Goszczynski and Jooste, 2002, 2003 a, b). Goszczynski and Jooste (2003b) showed that the primer pair H587/C995 could be used to study the variability of the coat protein gene among GVA groups I and II. Therefore, this primer pair was used to amplify a fragment of 430 bp from the coat protein gene of GVA. The total RNA extraction protocol described by Sambrook and Russell (2001) was applied to extract the RNA from 16 grapevine samples. Immunocapture-RT-PCR, a modification of RT-PCR, has been used in many studies to detect plant viruses in woody plant (Wetzel *et al.,* 1992; Minafra and Hadidi, 1994; Chevalier *et al.,* 1995). IC-RT-PCR is 500 and 1000 times more sensitive than DAS-ELISA in the detection of PPV and GVA respectively.

Although the samples used for the RT-PCR and the IC-RT-PCR reacted positively with the IgG specific for GVA in the DAS-ELISA test, the expected size of the CP gene could not be amplified from some GVA-ELISA positive samples. These results are not in accordance with the fact that RT-PCR is a more sensitive technique than DAS-ELISA (Wetzel *et al.,* 1992; Minafra and Hadidi 1994 and Chevalier *et al*., 1995). It is important to note that Goszczynski and Jooste (2003b) reported that the primer pair (H587/C995) used in this study is variant-specific, detecting only GVA isolates that belong to group I and II. It is therefore recommended to use a general primer pair that can detect all members of GVA from groups I, II, and III. The sequencing results of the GVA CP (430 bp) (data not shown) showed that all GVA isolates shared 100% nucleotide sequence similarity. In addition, the sequences of the GVA isolates reported in this study showed 100% nucleotide homology with the GVA-Jo (AY594176) described recently by Anfoka *et al*. (2004). These results suggest that a region other than the CP gene in the GVA genome must be investigated to differentiate between the Jordanian isolates of GVA. A suitable region may include the movement protein gene.

In the light of the alignment analysis, isolate GVA-5R could be classified as a member of GVA group I since it shared high nucleotide and amino acid sequence similarities with the members of this group. In conclusion, the data presented in this study showed that the GVA isolates collected from different locations in Jordan between 2004 and 2005 were identical at the molecular level. Attempts should now be made to investigate the population diversity among GVA isolates in certain locations and during several years, taking into consideration the size of the sample and the regions on the virus genome that should be subjected to sequence analysis.

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