

DETECTION AND MOLECULAR CHARACTERIZATION OF GRAPEVINE FANLEAF VIRUS AND GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 IN JORDAN

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SUMMARY

Grapevine fanleaf virus (GFLV) and *Grapevine leafroll-associated virus 3* (GLRaV-3) have been detected by Double-Antibody Sandwich (DAS)-ELISA in six locations where grapevines are commonly grown in Jordan. Using pairs of specific primers, fragments of the RNA-dependent RNA polymerase (*RdRp*) gene of GLRaV-3 and the coat protein (*CP*) gene of GFLV were amplified from symptomatic grapevine tissues by Immunocapture-Reverse Transcriptase-Polymerase Chain Reaction (IC-RT-PCR). After cloning and sequencing, sequences of the amplified fragments were deposited in Genbank. Alignment analysis revealed that the amplified *CP* gene fragment of the Jordanian isolate of GFLV (GFLV-JOR) shared sequence identity between 82 and 88% with 11 GFLV isolates from different parts of the world. High sequence identity (95-99%) was observed between the amplified fragment of *RdRp* gene of GLRaV-3 from Jordan (GLRaV-3-JOR) and isolates from Brazil, USA, Czech Republic and China.

Key words: grapevine, DAS-ELISA, IC-RT-PCR, GLRaV-3, GFLV.

INTRODUCTION

Diseases caused by *Grapevine fanleaf virus* (GFLV) and *Grapevine leafroll-associated virus 3* (GLRaV-3) have been reported in many grapevine-growing areas in the world. GFLV belongs to the subgroup A of the genus *Nepovirus* of the family *Comoviridae* (Wellink *et al.*, 2000) and is transmitted by the nematode *Xiphinema index* (Belin *et al.*, 2001). The most damaging consequence of fanleaf disease is a severe reduction in fruit setup that can greatly reduce the yield (Raski *et al.*, 1983). GFLV has a bipartite RNA genome (RNA1 and RNA2) of positive-sense, single stranded, polyadenylat-

ed RNAs, in addition to smaller satellite RNA (RNA3) in some isolates (Pinck *et al.*, 1988).

GLRaV-3 belongs to genus *Ampelovirus* of the family *Closteroviridae* (Martelli *et al.*, 2002) and it is phloem-limited. Disease symptoms caused by GLRaV-3 appear in *Vitis vinifera*, but they are latent in almost all American *Vitis* spp. Diseased leaves are thicker than normal, brittle with margins rolled downwards and discolored (Martelli, 1993). These symptoms are usually associated with yield losses of 20 to 40% (Woodrum *et al.*, 1984). GLRaV-3 is transmitted by mealybugs and scale insects (Rosciglione and Gugerli, 1989) and has a single stranded positive-sense genomic RNA (Ling *et al.*, 1998). It includes 13 open reading frames (ORF). ORF1 encodes the helicase protein from ORF1a, and the RNA-dependent RNA polymerase (*RdRp*) protein from ORF1b via a +1 ribosomal frameshift mechanism (Ling *et al.*, 1998).

Enzyme Linked Immunosorbent Assay (ELISA) has been considered as a reference method for routine virus diagnosis in grapevine. However, problems are still encountered in the detection of GLRaV-3 (Acheche *et al.*, 1999). Since closteroviruses can be distributed unevenly throughout the plant and their titers can vary considerably according to seasonal influences (Habibi *et al.*, 1997), detection based on molecular techniques is likely to be more sensitive. Several reports show the successful detection of closteroviruses by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) (Minafra and Hadidi, 1994; Acheche *et al.*, 1999).

Although GFLV and GLRaV-3 were previously reported to occur in Jordan (Savino *et al.*, 1975; Al-Tamimi *et al.*, 1998; Abu Shirbi, 2001), information on these viruses at the molecular level is not available. Therefore, the aims of this study were to investigate the distribution of GFLV and GLRaV-3 in grapevine-growing areas in Jordan and to characterize them at the molecular level.

MATERIALS AND METHODS

Sample collection. Leaf samples were collected from six different sites (Research Station of Al-Balqa' Applied University (BAU), Ajlun, Al-Salt, Al-Ramtha, Al-

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Mafrag and Jordan Valley) where grapevines are commonly grown in Jordan (Table 1). A total of 109 samples were collected from grapevines showing clustered foliage, vein banding and clusters with large and small berries, and tested for GFLV infection. Another 91 leaf samples with disease symptoms similar to those caused by GLRaV-3 were collected and tested for GLRaV-3 infection. All samples were placed in plastic bags and kept at 4°C until use.

Double-Antibody Sandwich (DAS)-ELISA. To detect GLRaV-3, leaf samples were prepared by peeling 0.5 g of midribs and main veins and homogenized 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). To detect GFLV, the whole leaf tissues were homogenized as mentioned above and extracts were clarified by centrifugation at 14,000 g for 5 min and kept on ice. DAS-ELISA was performed following the general protocol of Clark and Adams (1977) and the polyclonal antibodies against GLRaV-3 and GFLV, developed by Sediag (SEDIAG S.A, Strasbourg, France), were used according to the manufacturer's instructions. Each sample was analyzed in at least two wells, and mean experimental readings at least three times the mean reading of the negative controls were considered positive. Samples that showed high ELISA values were selected for IC-RT-PCR analysis.

Immunocapture-Reverse Transcriptase-Polymerase Chain Reaction (IC-RT-PCR). Samples to be used for IC-RT-PCR were extracted as indicated previously for DAS-ELISA. PCR tubes were precoated with 200 µl of diluted anti-GLRaV-3-IgG or anti-GFLV-IgG and incubated at 37°C for GLRaV-3 and at room temperature for GFLV for 4 h. Tubes were rinsed with PBS-Tween and 200 µl of extracts from GLRaV-3-infected, GFLV-infected or healthy grapevines, were added and tubes were in-

cubated at 4°C for 16 h. After washing with PBS-Tween, tubes were incubated at 65°C for 10 min and then kept on ice. RT-PCR was performed using the Access RT-PCR System (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. The primer pair C547 (5'-ATTAAGCTTGACGGATGGCACGC-3') and H229 (5'-ATAAGCATTTCGGGATGGACC-3') (Minafra and Hadidi, 1994) was used to amplify a fragment (340 bp) of the *RdRp* gene of GLRaV-3. The following thermal cycling scheme was used for 35 reaction cycles in a PTC200 type thermocycler (MJ Research Inc., Watertown, MA, USA): one cycle at 94°C of 5 min, then 15 cycles of 94°C for 50 sec, 54°C for 1 min, and 72°C for 1 min, followed by additional 20 cycles of 94°C for 50 sec, 46°C for 70 sec, and 72°C for 1 min. A final extension cycle at 72°C for 10 min ended the run (N. Iraki and O. Dar-Issa, personal communication). For GFLV, a fragment (606 bp) of the coat protein (*CP*) gene was amplified using the primer pair C2647 (5'-GTGAGAG-GATTAGCTGGT-3') and H2042 (5'-AGCACTCC-TAAGGGCCGT-3') according to Fattouch *et al.*, (2001). PCR products were resolved by 1.2% agarose gel electrophoresis and visualized after ethidium bromide staining with a UV transilluminator. DNA molecular weight markers (Promega Co., Madison, WI, USA) were used to determine the size of the amplified fragments.

Cloning and sequencing. PCR products were ligated to the pGEM[®]-T Easy Vector (Promega Co., Madison, WI, USA) and cloned according to the manufacturer's instructions. Recombinant plasmids were isolated from transformed *Escherichia coli* JM109 according to Sambrook and Russell (2001) and screened by restriction digestion with *EcoR*I followed by electrophoresis in 1.2% agarose gel. A clone of GLRaV-3 and GFLV was taken for sequencing and sequences were checked for homologous sequences available from the Genbank using the DNAMAN software program (Lynnon BioSoft, Que., Canada).

Table 1. Detection of *Grapevine leafroll virus 3* (GLRaV-3) and *Grapevine fanleaf virus* (GFLV) in different locations in Jordan by DAS-ELISA.

Location	<i>Grapevine leafroll virus 3</i> (GLRaV-3)		<i>Grapevine fanleaf virus</i> (GFLV)	
	No. of positive samples/ No. of tested samples	Infection rate (%)	No. of positive samples/ No. of tested samples	Infection rate (%)
Research Station of BAU	3/35	8.6	6/41	14.6
Ajlun	4/17	23.5	1/25	4.0
Al-Salt	4/7	57.0	0/7	0.0
Al-Ramtha	6/15	40.0	3/15	20.0
Al-Mafrag	4/5	80.0	0/9	0.0
Jordan Valley	8/12	66.7	1/12	8.3
Total	29/91		11/109	

RESULTS

GFLV and GLRaV-3 were detected by DAS-ELISA in symptomatic leaf samples. As shown in Table 1, GLRaV-3 was detected in all 6 locations. The highest rate of infection was recorded in Al-Mafraq (80%) and Jordan Valley (66.7%), whereas, only 8.6% of samples collected from the Research Station of BAU were infected. On the other hand, GFLV could be detected in only 11 samples (9.6%). The infected samples were from the Research Station of BAU, Al-Ramtha, North Jordan Valley, and from Ajlun. While no GFLV infection could be detected in samples collected from Al-Salt, or Al-Mafraq areas.

Detection of GLRaV-3 and GFLV by IC-RT-PCR.

Parts of the CP gene (606 bp) of GFLV from Jordan (GFLV-JOR) and the RdRp gene (340 bp) of the Jordanian isolate of GLRaV-3 (GLRaV-3-JOR) were amplified from infected grapevine tissues using the primer pairs C2647/H2042 and C547/H229, respectively (Fig. 1). No band could be detected when leaf extracts from healthy grapevines were used. The identity of the amplicons was confirmed by sequencing.

Cloning and sequencing. Sequencing results confirmed that the PCR products obtained by IC-RT-PCR were indeed fragments of the CP gene of GFLV and the RdRp gene of GLRaV-3. The sequences of the amplified fragments were deposited in the Genbank under accession no. AY594177 for the CP gene of GFLV-JOR and AY628766 for the RdRp gene of GLRaV-3-JOR. Comparison analysis of the cloned fragment of the CP gene of GFLV-JOR revealed a low degree of identity (e.g. 82-88% nucleotide identity and 91-96% amino acid similarity) with other GFLV isolates (Table 2). On the other hand, the nucleotide sequence of the amplified RdRp gene of GLRaV-3-JOR had an identity match of 99%

with published sequences of three GLRaV-3 isolates (Table 3), and the four isolates were identical at the amino acid level. The lowest nucleotide identity (95%) was observed with GLRaV-3 isolate from Brazil (accession no. AF438411).

DISCUSSION

To our knowledge, data shown in this study represent the first report on the characterization of GLRaV-3 and GFLV at the molecular level in Jordan.

As shown in Table 1, GLRaV-3 could not be detected in all symptomatic leaf samples. A possible explanation is that samples were collected from grapevines that were infected with other closteroviruses that induced similar disease symptoms (Boscia *et al.*, 1995). Similarly, only a limited number of samples that showed grapevine fanleaf disease symptoms tested positive by DAS-ELISA for GFLV (Table 1). This might be due to the uneven distribution of the GFLV within the infected trees (Abu Shirbi, 2001). In addition, observed symptoms might be due to infection with other viruses, such as *Arabis mosaic virus* (Loudes *et al.*, 1995).

Data from this study show that IC-RT-PCR is a reliable technique and can be used routinely to detect GLRaV-3 and GFLV directly from grapevine tissues (Fig. 1). This is in accordance with Acheche *et al.* (1999) who showed that IC-RT-PCR is an effective and practical technique to screen grapevines and viruliferous mealybug vectors for the infection with GLRaV-3.

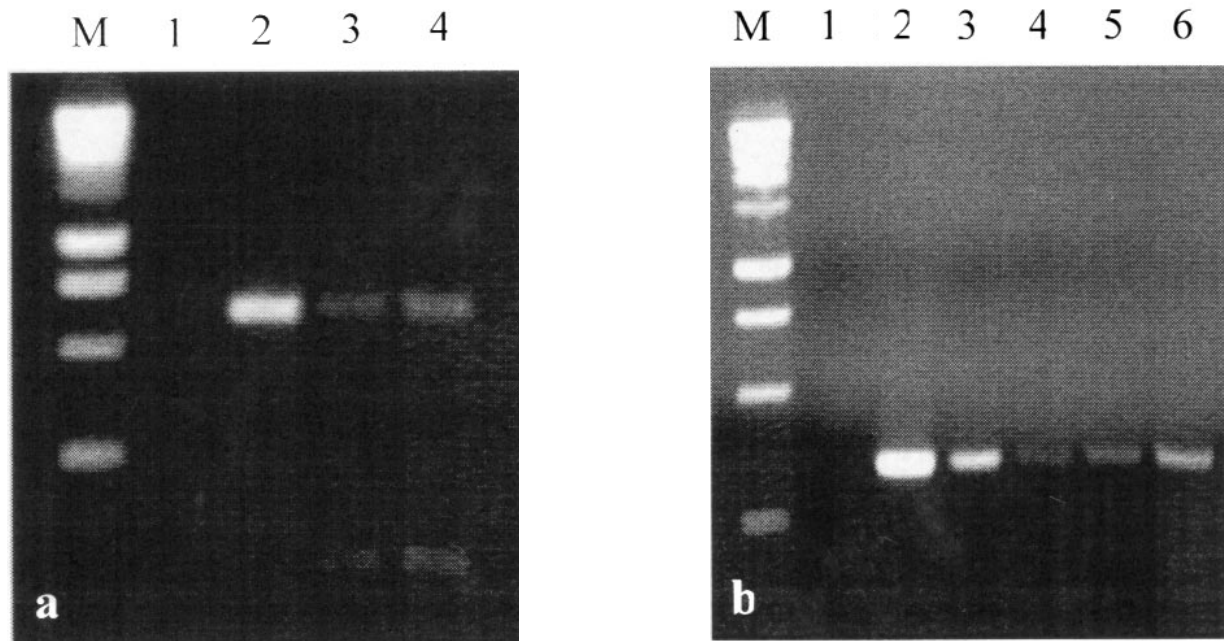
The fragments of CP and RdRp genes were directly amplified from natural host plant and could therefore be regarded as more definitive than sequences of fragments amplified from herbaceous host. Naraghi-Arani *et al.* (2001) showed that the sequence of the CP gene of GFLV could be altered with passage through the alternative host *Chenopodium quinoa*, suggesting that genomic

Table 2. Comparison of sequence similarity of the amplified coat protein (CP) gene fragment of *Grapevine fanleaf virus* (GFLV) from Jordan (GFLV-JOR) and GFLV isolates from other countries.

Isolates	Origin	Accession no.	Nucleotide identity (%)	Amino acid similarity (%)
F13	France	X16907	88	96
Fanleaf	Spain	X60775	88	93
Hangzhou	China	AJ318415	87	94
NW	Germany	AY017338	86	95
Livermore Block 25	USA	AF304014	86	92
GFLV-FC	Austria	U11768	85	94
TunSp1-1	Tunisia	AY525605	84	91
GFLV108	USA	AF304013	84	93
Walker	USA	AF304015	84	92
RS	Brazil	AF418579	84	97
TunSp2-1	Tunisia	AY525606	82	91

Table 3. Comparison of sequence similarity of the amplified RdRp gene fragment of *Grapevine leafroll-associated virus 3* from Jordan (GLRaV-3-JOR) and GLRaV-3 isolates from other countries.

Isolates	Origin	Accession no.	Nucleotide identity (%)	Amino acid similarity (%)
Not known	China	AY495340	99	100
Muller-Thurgau	Czech Republic	AY424407	99	100
NY1	USA	AF037268	99	100
Not known	Brazil	AF438411	95	96

**Fig. 1.** Agarose gel electrophoretic analysis of the amplified fragments of the coat protein (CP) (a) and RNA dependent RNA polymerase (RdRp) (b) genes of GFLV-JOR and GLRaV3-JOR, respectively. M, 1-kb DNA size marker; lane 1, healthy control; lanes 2, 3, 4, 5 and 6 symptomatic field samples.

variability should be characterized directly in the primary host. The sequenced part of RdRp gene of GLRaV-3-JOR showed high degree of identity with other GLRaV-3 isolates from different parts of the world (Table 3) suggesting that they are closely related. This is in accordance with results reported by Ling *et al.* (1998) that the RdRp gene is conserved among closteroviruses. Sequence similarity analyses of the amplified CP gene fragment suggest that GFLV-JOR might be a different isolate from isolates mentioned in Table 2. However, these results should be confirmed by sequencing the complete CP gene or other genes of the GFLV-JOR genome.

Data presented in this study provide new information on the molecular constituents of two important grapevine viruses that cause high yield losses of grapevines in Jordan. However, much effort is needed to study the occurrence and distribution of other grapevine virus and virus-like diseases. In addition, the response of

local as well as the imported grapevine cultivars to the infection with GFLV and GLRaV-3 should be investigated.

ACKNOWLEDGEMENTS

This research was supported in part by the Middle East Research and Cooperation (MERC) project M21-037. The authors would like to thank Mr. Mohammad Abhary for his technical assistance during this study.

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