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RESEARCH PAPERS

Prevalence and genetic diversity of *Grapevine virus A* in Tunisia

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Summary. Prevalence and genetic diversity of the complete CP gene of *Grapevine virus A* (GVA) were assessed in isolates from rootstocks, wine and table grape varieties and autochthonous grapevines. Wine grapes were the most infected (63%), followed by table grapes (49%) and rootstocks (44%). Autochthonous grapevines were the less infected (35%). Analyses of the complete coat protein sequences of 20 GVA isolates from the main grapevine growing areas of Tunisia identified three phylogroups, accounting, respectively, for 70% (group I), 25% (group IV) and 5% (group III) of the isolates. No sequences clustered into group II. Phylogenetic analyses indicated that Tunisian GVA isolates are not grouped by the host cultivar or geographic origin.

Key words: Grapevine, GVA, molecular analysis, diversity, isolates.

Introduction

Rugose wood (RW) is one of the major disease complexes affecting grapevines (*Vitis* species) (Martelli and Boudon-Padieu, 2006). RW affects woody grapevine stems, causing pitting, grooving and other distortions (Martelli, 1993). Symptom severity varies according to the rootstock-scion combinations, but latency in infected grapevines is also often observed. Affected grapevines show reduced vigour and growth, decline and, in the more severe cases, they die within a few years from planting (Meng *et al*., 1999). RW-affected grapevines can show marked differences in the diameter of their scions and rootstocks at the graft unions, with associated reductions in crop yield and fruit quality (Golino *et al*., 2000). According to the symptoms induced on woody indicator plants, four syndromes of RW complex can be distinguished, associated with different viruses. These are; Rupestris stem pitting, LN 33 stem grooving, Corky bark, and Kober stem grooving.

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Grapevine virus A (GVA) has been implicated with Kober stem grooving (Garau *et al*., 1994) and Shiraz disease in South Africa and Australia (Goszczynski and Habili, 2012). This virus belongs to the family *Betaflexiviridae,* genus *Vitivirus*, and has flexuous, filamentous particles of length approx. 800 nm (Minafra *et al*., 1997). The genome is organised into five open reading frames (ORF) encoded by a positive strand RNA of 7351-7471 nt (Goszczynski, 2014). ORF1 encodes replication-related proteins; ORF2 encodes a protein of 19 kDa, with unknown function; ORF3 encodes a putative movement protein and ORF4 encodes the coat protein (CP). ORF5 encodes a small protein of 10 kDa, with unknown function which may be a putative nucleic acid binding protein (Galiakparov *et al*., 2003; Minafra *et al*., 1997). Based on sequence analyses of the complete CP gene, groups I, II, III and IV were reported (Murolo *et al*., 2008; Wang *et al*., 2012; Alabi *et al*., 2014). Isolates in group III were found to induce mild symptoms, while group I and II isolates induced more severe symptoms in *Nicotiana benthamiana* (Goszczynski and Jooste, 2003a). Mixed infections by divergent variants of GVA are

common in South African vineyards (Goszczynski and Jooste, 2003b), where the majority of GVA variants in group II were consistently associated to Shiraz disease, while variants in group III were associated with asymptomatic grapevines susceptible to the Shiraz disease (Goszczynski, 2007; Goszczynski *et al*., 2008). GVA is usually disseminated in the vineyards through infected propagation material, and can be spread by mealybugs and soft scales, often together with leafroll-associated viruses (Rosciglione and Castellano, 1985; La Notte *et al*., 1997; Zorloni *et al*., 2006; Le Magu et *et al*., 2012; Bertin *et al*., 2016). GVA can play a key role in the development of RW disease. The virus is one of the most common grapevine viruses in table and wine grapes in Tunisia, butno studies have been carried out on diversity of GVA in this country. This paper describes the distribution and the genetic diversity of GVA isolates, carried out to determine their population structure in relationship to cultivars and their geographic origins.

Materials and methods

Sample collection

Field surveys were carried out in the main grapevine growing regions of Tunisia (Cap Bon, Mornag, Rafraf and Bousalem). A total of 403 samples were collected, comprising 141 from autochthonous grapevine varieties maintained in a germplasm collection established at INRAT (Tunis), 115 samples of wine grape and 115 of table grape varieties from commercial vineyards, and 32 from rootstocks from mother plots. Mature canes were randomly collected in winter and stored at 4°C, until laboratory testing.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was used to test all the samples for the presence of GVA, using specific primers H7038/C7273 (Mackenzie, 1997) for the amplification of a 236 bp fragment of the CP gene.

Total nucleic acids extraction

Total nucleic acids (TNA) were extracted according to Foissac *et al*. (2001). About 0.2 g of phloem tissues (cortical scrapings) from each sample were ground in 1 mL of extraction buffer (4 M guanidine thiocyanate, 0.2 M sodium acetate ($C_2H_3NaO_2$) pH 5.2, 25 mM EDTA, 1.0 M potassium acetate ($C_2H_3KO_2$) pH 5.0 and 2.5% w/v PVP-40), and then mixed with 2% sodium metabisulfite as antioxidant. The mixture was transferred into an Eppendorf tube containing 100 µL N-Lauroylsarcosine sodium salt (10%) and incubated at 70°C for 10 min, then placed on ice for 5 min. After centrifugation at 13,000 rpm for 10 min, 300 µL of supernatant was transferred to an Eppendorf tube to which were added 150 µL absolute ethanol, 300 µL of 6M sodium iodide (Nal) and 50 µL of SiO_2 solution (12%, pH 2.0). The mixture was stirred for 30 min at room temperature and then centrifuged at 6,000 rpm for 1 min. The pellet was recovered and washed with 500 µL of washing buffer [50% STE 1× (10mM Tris-HCl, pH 8.0, 1mM EDTA and 100mM NaCl), 50% absolute ethanol]. It was then re-suspended in 120 µL of sterile distilled water, incubated for 3 min at 70°C and then centrifuged at 13,000 rpm for 3 min. The supernatant was transferred to a new Eppendorf tube and stored at -20°C.

Reverse transcription and amplification

Five hundred ng of TNA extracts were mixed with 1 µL random primers (1 µgµL^{-1}) and 1.5 µL of sterile water and denatured at 95°C for 5 min. Reverse transcription was done for 1h at 39°C in 1 µL Moloney Murine Leukemia Virus M-MLV (200 UµL^{-1}) (Invitrogen Corporation), 4 µL buffer (5×First-strand), 2 µL DTT (0.1 M) and 0.5 µL dNTPs (10 mM),adjusted to a final volume of 25 µL with sterile distilled water. A volume of 2.5 µL of the synthetized cDNA was submitted to PCR amplification using a mixture containing 2.5 µL 10×Taq polymerase buffer, 1 µL $MgCl_2$ (50 mM), 1 µL dNTPs (10 mM), 0.5 µL of each primer GVA-H7038 and GVA-7273 (20 µM) and 0.25 µLTaq polymerase (5 UµL^{-1}) (Invitrogen Corporation). The mixture was adjusted to a final volume of 25 µL with sterile distilled water. PCR reactions consisted of one cycle at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 35 sec, annealing at 52°C for 45sec, elongation at 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gel in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3), and visualized under UV light after staining with ethidium bromide.

Sequencing and sequence analyses of the coat protein gene

Twenty GVA isolates were chosen for the amplification of the full length CP gene (740bp), using spe-

Table 1. List and identifiers of *Grapevine virus A* isolates sequenced and analyzed in this study.

Isolate	Cultivar	Origin	Coat protein gene Accession number
CR8	Carignan	Takelsa	LT900345
CR11	Carignan	Takelsa	LT900346
CR15	Carignan	Grombalia	LT900347
D40	Dabouki	Takelsa	LT900348
MI45	Italia	Takelsa	LT900349
MI51	Italia	Takelsa	LT900350
MI54	Italia	Baddar	LT900351
MI55	Italia	Baddar	LT900352
MI56	Italia	Baddar	LT900353
MI59	Italia	Grombalia	LT900354
MI60	Italia	Grombalia	LT900355
AV16	Autochthonous	Rafraf	LT900356
AV27	Autochthonous	Kef	LT900357
AV28	Autochthonous	Kef	LT900358
AV55	Autochthonous	Gabes	LT900359
CR011 34	Carignan	Grombalia	LT900360
GN121 94	Grenache	Grombalia	LT900361
GN0855	Grenache	Kelibia	LT900362
GN1267	Grenache	Kelibia	LT900363
AV130	Autochthonous	Rafraf	LT900364

cific primersGVA-CPF6356and GVA-CPR7096 (Alabi *et al*., 2014). Isolates were from the major cultivated table grape and wine grape cultivars as well as from autochthonous grapevines from different Tunisian regions (Table 1).

PCR products of these GVA isolates were purified with the ExoSAP-IT purification kit (Affymetrix). Direct sequencing was done with the same primers used for RT-PCR on a 3730xl DNA analyser automated sequencer (Applied Biosystems), and sequences were submitted in the European Nucleotide Archive (ENA; www.ebi.ac.uk/ena). Sequences of Tunisian isolates provided from this study were compared to selected international strains retrieved from GenBank, using MEGA7 software (Kumar *et al*., 2016). In order to assess evolutionary relationships in the Tunisian GVA population, phylogenetic analyses were performed by neighbour joining methods, with 1,000 bootstrap replicates and using the same software. *Grapevine leafroll associated virus* 3 (GenBank accession number JX088242.1) was used as an out group.

Recombination analyses

Recombination analyses using the obtained alignment were performed with the genetic algorithms for recombination detection (GARD) available on the Data monkey webserver (Kosakovsky Pond *et al*., 2006).

Results

GVA prevalence

RT-PCR assays successfully amplified the expected product from 48% of the tested samples. Wine grapes were the most infected (63% of wine grape samples), followed by table grapes (49%) and rootstocks (44%) (Table 2). Autochthonous grapevines were the least infected (35%). The most cultivated wine cultivars Carignan and Grenache had infection rates of, respectively, 80 and 57%. For table grapes, cv. Italia which includes almost 70% of the Tunisian table grape area had an infection rate of 75%. For rootstocks, 110R was the most infected (54%), followed by 1103P (40%) and 140Ru (33%) (Table 2).

Phylogenetic analysis

The comparison at nucleotide and amino acid levels of the 20 sequenced GVA isolates indicated that the similarity among Tunisian isolates ranged from 77% to 98% at the nucleotide level and 75 to 98% at the amino acid level (Table 3). The isolates CR8, CR15, D40, MI45, MI54, MI55, MI60, AV16, AV27, AV55, CR011 34, GN0855, GN1267 and AV130 showed nucleotide similarity ranging from 90 to 98%. Among these isolates AV16 and GN1267 showed nucleotide similarity of 98%, although they were from two different host varieties collected from two regions (Table 3). The most divergent isolates in this group (90% nucleotide similarity) were GN0855 and GN1267, collected from Grenache vines in one vineyard (Table 3). Five isolates CR11, MI51, MI56, MI59 and GN121 94 shared 90% to 96% nucleotide similarity and 92-97%

Table 2. Distribution of *Grapevine virus A*in Tunisian grapevine types and cultivars, and respective infection rates.

Grapevine type	Cultivar	Number of samples	Infection rate (%)
Winegrape	Grenache	56	80
	Carignan	42	57
	Alicante Boushet	12	75
	Others	5	60
		115	63
Table grape	Italia	36	75
	Red Globe	20	5
	Dabouki	15	33
	Rich Baba Sam	9	89
	Others	35	43
		115	49
Rootstock	1103P	10	40
	140Ru	9	33
	110R	13	54
		32	44
Autochthonous grapevine		141	35
Total		403	48

amino acid similarity. The most related isolates from one cultivar (Italia) were MI51 and MI56, and sharing 96% nucleotide similarity and 97% amino acid similarity. Isolate CR11 from cv. Carignan shared 92-94% nucleotide similarity with isolates MI51, MI56, MI59 and GN121 94. Isolate AV28 from an autochthonous cultivar collected from Kef (North West Tunisia) was distant from all other Tunisian sequences, with 77-82% nucleotide similarity and 75–81% amino acid similarity (Table 3).

To assess the phylogenetic relationships within the GVA population, sequences downloaded from the GenBank and Tunisian sequences were clustered. The resulting tree showed four distinct phylogroups (I, II, III and IV) (Figure 1), characterized by a within-group mean percent similarity ranging from 89% to 93%. Isolates from group IV had the greatest nucleotide similarity (93%), followed by group II (91%) and group I (90%). Isolates from the group III had the least nucleotide similarity (89%). The between group mean percent similarities ranged from 78% to 88%. Group I shared 85% nucleotide similarity with group II, 88% with group IV and 78% , with group III. Group II shared 86% similarity with group IV and 78% similarity with group III. Group III shared 78% nucleotide similarity with all the other groups.

The 20 Tunisian GVA isolates belonged to three of the four described phylogenetic groups. Fourteen isolates (CR8, CR15, D40, MI45, MI54, MI55, MI60, AV16, AV27, AV55, CR011 34, GN0855, GN1267 and AV130) clustered into the group I (Figure 1). Five isolates (CR11, MI51, MI56, MI59 and GN121 94) clustered into the group IV, and one isolate (AV28) clustered into the group III. None of the 20 isolates clustered into the group II. Isolates from autochthonous grapevines clustered with isolates from cultivated varieties into group I, with the exception of isolate AV28 which clustered into group III (Figure 1).

Isolates from the table grape cv. Italia clustered into group I (MI45, MI54, MI55 and MI60) and group IV (MI51, MI56 and MI59). Isolates collected from the one cultivar and one vineyard clustered in different groups, such as isolates MI45 and MI51collected located at Takelsa, MI55 and MI56from Baddar, and MI59 and MI60 from Grombalia. Four isolates collected from wine grape cv. Carignan, clustered into group I (isolates CR8, CR15 and CR01134) and group II (CR11). Three isolates from wine cv. Grenache grouped into group I (isolates GN0855 and GN1267) and group II (GN12194).GARD analyses implemented in the Data monkey webserver (www.datamonkey.org) found evidence of recombination breakpoints at the position of 285 of the affined alignment of 564nt. The Kishino Hasegawa topological incongruence revealed one breakpoint with a significant topological incongruence.

Discussion

The GVA infection rate (48%) observed in this survey for the tested grapevine samples is in accordance with previous studies reporting distribution of the virus in Tunisian grapevines (Mahfoudhi *et al*., 1998; 2014). The incidence of GVA is usually very high in the Mediterranean countries which have long grapevine growing traditions. Results obtained in Tunisian vineyards are close to those reported in Lebanon (32%) (Haïdar *et al*., 1996), Italy (41%) and Turkey (55%) (Di-

Table 3. Nucleotide (below diagonal) and amino acid (above diagonal) similarities (%) of CP genes of 20 Tunisian isolates of *Grapevine virus A*.

GVA isolate	CR8	CR11	CR15	D40	MI45	MI51	MI54	MI55	MI56	MI59	MI60	AV16	AV27	AV28	AV55	CR011 34	GN121 94	GN0855	GN1267	AV130
CR8	100	92	96	95	93	92	95	94	93	93	93	92	93	78	93	95	91	92	93	93
CR11	89	100	91	92	89	93	93	91	93	94	93	90	91	81	90	91	92	88	92	90
CR15	93	91	100	96	95	94	97	95	95	93	93	93	93	77	94	96	91	93	94	93
D40	91	89	92	100	96	95	96	96	95	95	97	95	94	77	95	96	93	93	96	95
MI45	91	87	92	94	100	93	95	96	93	91	95	95	93	75	95	95	90	93	95	93
MI51	89	92	91	94	90	100	94	93	97	96	96	92	92	79	93	93	95	92	94	92
MI54	92	91	94	94	92	93	100	95	93	95	95	94	96	78	95	97	93	95	96	96
MI55	92	89	94	93	93	90	93	100	94	93	95	93	93	76	95	94	92	91	93	93
MI56	89	92	91	94	90	96	92	90	100	95	95	92	92	78	93	93	93	91	93	91
MI59	90	91	90	94	91	95	93	90	94	100	97	90	93	79	91	95	98	92	92	92
MI60	91	90	92	94	94	93	93	94	92	95	100	93	94	77	93	94	96	91	95	93
AV16	90	89	90	90	90	89	92	91	90	89	90	100	93	78	98	93	90	92	98	93
AV27	90	88	91	90	90	89	91	91	88	89	91	88	100	80	93	95	93	92	95	98
AV28	79	79	78	79	78	79	79	78	79	78	77	78	82	100	77	78	79	79	79	78
AV55	91	88	91	90	91	90	93	92	90	89	90	96	89	79	100	94	90	94	98	94
CR011 34	92	88	93	92	93	90	94	93	90	92	93	90	91	78	90	100	93	95	94	95
GN121 94	89	90	90	91	90	93	92	90	92	96	94	89	89	78	89	91	100	90	92	92
GN0855	90	87	91	91	91	89	93	92	89	90	91	89	90	79	91	95	90	100	94	92
GN1267	90	89	90	90	90	90	93	91	90	90	90	98	88	78	96	89	89	90	100	95
AV130	91	88	91	91	91	89	92	91	88	90	91	89	97	81	90	92	90	91	89	100

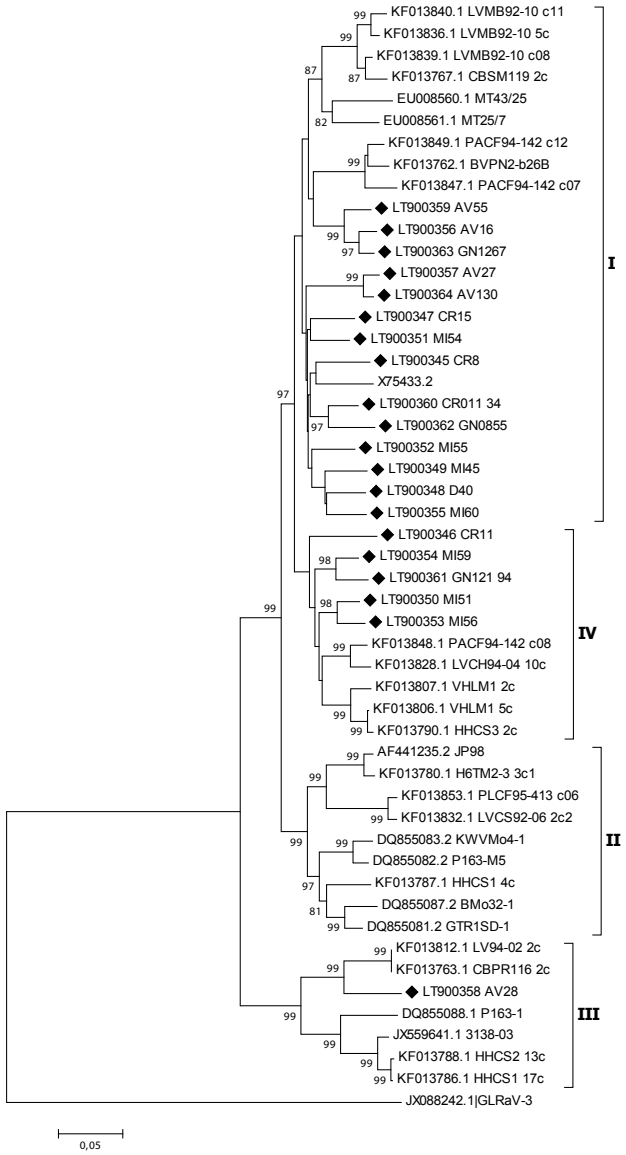

Figure 1. Phylogenetic tree of 20 isolates of *Grapevine virus A* isolates from Tunisia (indicated by ♦), constructed with 36 sequences of a 590nt fragment from the CP gene region, obtained in this study and those retrieved from the GenBank. Percentage of bootstrap support (≥75%) from 1,000 replicates. *Grapevine leafroll associated virus* 3 (GenBank accession number JX088242.1) was used as an outgroup.

giaro *et al*., 1999), Palestine (66%) (Alkowni *et al*., 1998), Egypt 68% (Ahmad *et al*., 2004) and Morocco (more than 80%) (Digiaro *et al*., 1999). Other studies have revealed low prevalence of GVA in South Africa (19%) (Jooste *et al*., 2015), Malta (12%) (Digiaro *et al*., 1999), Kosovo (11%) (Dida *et al*., 2012), Portugal (6%) (Digiaro *et al*., 1999), Russia (6%) (Porotikova *et al*., 2016) and China (5%) (Fan *et al*., 2013). The high GVA infection rate in mother plants used for bud production (Mahfoudhi *et al*., 1998) and the common presence of putative vectors in the field, including *Planococcus ficus* (Signoret) and *Planococcus citri* (Risso) (Mahfoudhi and Dhouibi, 2009), explains the common occurrence of GVA infections in Tunisian vineyards.

This is the first reported study of the genetic diversity of GVA in Tunisian grapevines, based on the comparison of the complete CP gene at both nucleotide and amino acid levels. The phylogenetic analysis showed that Tunisian GVA isolates are in three main phylogroups, with most sequences included in group I (14/20). This is in accordance with previous studies in Slovakia (Predajňa and Glasa, 2016) and Italy (Murolo *et al*., 2008). Group IV (25%) was the second most prevalent group, and this is different from all previous studies, where isolates from this group are less common. Only one isolate from a Tunisian autochthonous cultivar clustered in Group III, while in California and Washington States of the USA, this group was the most prevalent (Alabi *et al*., 2014). No Tunisian sequences clustered into group II. Isolates collected from the cultivar Italia in one vineyard clustered in different groups. This could be due to the use of grapevine propagating material infected with different isolates, or have arisen from between and within vineyard transmission of different GVA isolates by mealybug vectors.

A possible explanation of the presence of GVA isolates clustered in the same group but in different grape cultivars planted in distinct geographical locations, is that the initial source of cuttings of these cultivars were derived from scion material grafted onto common rootstocks infected with the same isolate. Tunisian GVA isolates cannot be separated according to the host cultivar or their geographic origin. Similar results were found in previous studies in other countries (Alabi *et al*., 2014; Murolo *et al*., 2008; Predajňa and Glasa, 2016).

The divergence within and between group similarities indicates that further study is warranted of the effects of nucleotide divergence on biological properties of GVA. Furthermore, since a single host plant may be infected by several variants of GVA, the use of advanced techniques for wide genetic variant screening of GVA should be implemented. Recombinant event analyses showed that GARD analyses

implemented in the Data monkey webserver found evidence of recombination breakpoints. This lead to evolution of this virus by genome exchange between different variants in a mixed infection system. Alabi *et al*. (2014) reported recombinant event within the GVA population allowing genetic exchange and development of new genetic variants.

Although the results of the present study showed no correlation between the phylogenetic position of viral isolates and geographic distribution of grapevine cultivars, more investigation is required to assess the prevalence and the distribution of specific viral genotypes. In particular, study of autochthonous cultivated and wild grapevines is warranted, to identify putative sources of new genetic variants which may present different biological properties (such as virulence and transmissibility), because different symptoms were obtained for variants of groups I, II and III when transmitted to *Nicotiana benthamiana* (Goszczynski *et al*., 2008).

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