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Research article

Analysis of the grapevine fanleaf diseaseand genetic diversity of tunisianGFLV Isolates

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Abstract

Genetic analyses of grapevine fanleaf virus (GFLV) was done on the basis of the coat protein gene sequences from 11 isolates detected in 3 tunisian grapevine growing regions:Grombalia, Rafraf andTakelsa and amplified with a newly designed primer pairGT 1076/GT 1826.The amplified region of 2^{CP} gene(750pb) wascaracterised by RT-PCR. After sequencing, alignement shows a variability within the obtained isolatesranged from 8.1 % to 15.4 % at the nucleotide level . Based on the result of genetic divergence of 312 pb long part of the 2^{CP} gene, the level of variation found in this study suggests that this rate of diversity is common for each gene of the coat protein.

Key words: *Grapevine, GFLV, symptomology, genetic diversity.*

1. Introduction

Grapevine fanleaf virus (GFLV),a*Nepovirus*of the Secoviridaefamily (Sanfaçon*et al.*, 2009), is one of the oldest viral diseases of grapevines (Martelli, 1986) . It is transmitted by the ectoparasitic root nematode *Xiphinema index*(Esmenjaud*et al.,* 1993)andthrough vegetative propagation and grafting (Zhou*et al*., 2015). The genome of GFLV is composed of two positive sense single-stranded RNA molecules each coding for a polyprotein and containing one open reading frame (ORF)(Pinck*et al*., 1988). The size of RNA1 is 7.342 nucleotides and RNA_2varies between 3.774 and 3.806 nt. The polyprotein encoded by RNA_1(P1)is processed into 5 proteins including a putative proteinase cofactor, a putative helicase5 ($1B^{Hel}$),a virus genome linked protein ($1C^{VPG}$), cysteine proteinase($1D^{Pro}$) anda putative RNA-dependent RNA polymerase ($1E^{Pol}$)(1). RNA_2 encoded protein P2 contains 3 proteins including homing protein($2A^{HP}$), movement protein ($2B^{MP}$) and coat protein($2C^{CP}$)(Andret-Link*et al.,* 2004).GFLV is a devastating viral disease it has been reported in several countries all over the world with 96%, 71%, 50%,24% of infection respectively in Spain (Bertolini*et al*., 2010),Tunisia(Mrabet*et al.,* 2015), Switzerland (Reynard and Guergli, 2012) and Chile (Poljua*et al*., 2010). In order to develop suitable strategies for controlling this virus, it is important to understand its genetic diversity and way of transmission. Severalstudies have assessed various molecular variants of GFLV in France (Vigne*et al*., 2004a),USA (Mekuria*et al*., 2009), Africa (Liebenberg*et al*., 2009), Iran(Bashir and Hajizadeh,2007) and Tunisia (Fattouch et *al.,* 2005).These studies focused mainly on $2C^{CP}$(coat protein) geneswhichare characterized by a strong genetic stability.In Tunisia genomic variability have been examined in several studies in the north by(Boulila, 2007; Fattouch et *al.,* 2005; Mrabet *et al*., 2015)where a divergence of 11%,14% and 15% was observed. The objective of this study was to deepen our knowledge of the genetic variability of the partial nucleotide sequence of the2^{CP} gene of GFLV in Tunisia,using new designed primers

2. Materials and Methods

2-1 Plant material

Thirty hundred samples were collected from major grapevine growing Tunisian regions;Grombalia, Mornag, Rafraf and Takelsa. Samples of phloem were collectedin January outside of the vegetative period and samples of leaves were collected in May during the vegetative period on two cultivars: Carignon and Muscat. All samples were placed in plastic bags and stored at +4°C for later use.

2-5 Double-Antibody Sandwich (Das-ELISA)

All samples were tested by Das-ELISA for the presence of GFLV. Leaves and cambial scarping of collected grapevine samples were ground in Bioreba bags in an extraction buffer prepared according to the Biorebaprotocol. Das-ELISA was carried using 1:1000 dilution of polyclonal antiserum anti-GFLV IGg. Absorbance was recorded at 405 nm using an automatic microplate reader (Multisacan Ascent, Labsystems USA). The threshold for detection was set to twice the average absorbance of the replications of healthy controls.

2-2 Extraction of total nucleic acid

Total nucleic acid from Das-ELISA positive samples was extracted based on silica-capture method (Foissac*et al.,* 2001). For this purpose,0.5 g of each sample was grinded with buffer (4.0 M guanidine thiocyanate, 0.2 M NaOAc, 2.5% PVP (40), 25 mM EDTA, 1.0 KOAc,0.2% Bisulfite de sodium). Extracts were transferred into new tubes involving 100ml of 10% Sodium lauryl sarcosyl solution. After homogenization, samples were incubatedat 70°C for 10 min and then in ice for 5 min. Each tube was centrifuged at 13000rpm for 10 min.300 µl of the supernatant are transferred to new eppendorfs containing 150µl of ethanol,300 µl of Sodium Iodide and 40%µl of Silica. The mixture was incubated on shaker for 30 min at room temperature. After centrifugation at 6000 rpm for 20 secs, supernatant was discarded. In order to wash silica particules,500 µl of washing buffer was added to each tube.120µl of free sterile water was next added to tubes which centrifuged at 13000 rpm for 10 min. The supernatant was finally transferred to new tubes and kept at -20°C.

2-3 cDNA synthesis

10 µl of TNA extractswere mixed with 1 µl random hexamer primer.8 to 10 µl of TNA extract were mixed with 0.5 µl random hexamers primer (Boehringer Mannheim, GbmH) (3 µg/µl) and 1.5µl of sterile water, denatured at 95°C for 5 min and kept in ice for 5 min. Reverse transcription was carried / performed for 1h at 39° in 1µl M-MLV, 4µl buffer 5x (50mM tris- HClph 8.3, 75mM KCl , 3mM $MgCl_2$), 2µl mM DTT and 1 mMdNTPs.

2-4 PCR

The amplification of partial region of the PC of GFLV was performed using a pair of universal primers (Table1) designated by MacKenzie*et al.*, 1997. The PCR reaction had the following mixture: 2.5 µl of cDNAtemplate, 2.5 µl of buffer 10x (Bioron), a final concentration of 1.5 mM $MgCl_2$, 0.5 µldNTPs (10 µM), 0.5 µl of each direction and antisense primer (10 µM)

and 0.25 µl of Taq polymerase (2.5 U). Amplification was performed in total volume of 25µl. The PCR program of amplification was used in a 36-well Rotor-Gene QIAGEN 5-Plex as follows: 94°C for 5 min followed by 35 cycles of 94°C for 30s, 55°C for 45s, 72°C for 1min.the final step is 72°C for 7 min.

Table1. List of primers used to amplify GFLV

Primer	Sequence	Position	Lengh
FL C3310	5'-GATGGTAACGCTCCCGCTGCTCTT-3'	3286-3310	312pb
FL H2999	5'-TCGGGTGAGACTGC GCAACTTCCTA-3'	2999-3024	

2-5 Primer design

Another pair of primers was designed in this study in order to amplify another part more larger of the PC region (750 bp) to study the genetic diversity of GFLV.First, global alignment using the software Geneious version 3.6.2 (http://www.geneious.com) (Kearse*et al.*,2012) was performed on partial CDS nucleic sequences of coat protein from several isolates of Grapevine fanleaf virus from Brazil,Chile,France, Iran and Italy retrieved from GenBank (Table 2) .Focus was made on highly conserved regions to pick out manually some primers which might amplify a larger region of the CP. Finally, a primer pair that should amplify specifically 750 bp sequence was selected (Table 3) regarding the assessment of the secondary structures made through the software Netprimer(http://www.premierbiosoft.com/netprimer/). Annealingtemperatures were also predicted using Netprimer.

2-6 PCR

The PCR mixture consists of 5µl of each cDNA template amplified in a reaction volume of 25µl containing 2.5µl of buffer 10x (Bioron), a final concentration of 1.5 mM $MgCl_2$, 0.5 µldNTPs (10 µM), 0.5 µl of each direction and antisense primer (10 µM) and 0.25 µl of Taq polymerase (2.5 U).
The PCR cycle conditions were: 94°C for 4 min, then 35 cycles of 94°C for 30s,57°C for 45s and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. Then, PCR products were run on1.2% agarose in 0.5*TAE buffer containing 2µl ethidium bromide.

2-7 Sequencing of PCR products

PCR products showing asingle band at the expected size, on agarose gel were sent to Macrogen, Inc. (Amsterdam, Netherlands) to be purified andsequenced.

2-8 Phylogenetic analyses

An alignment of 11 sequences of newly and previously reported isolates was made using ClustalWand implemented into Bioedit software.Nucleotides sequences identity levels were calculated using the same software.Phylogenetic tree was constructed using NJPLOT 2.3 (Perrière and Gouy, 1996).

Table 2 . List of isolates used to design primers

Accession	Isolate	Host	Origin
EU258681	IAC	106-8 greffé sur cv.IAC766	Brasil
EU258680	RUP	IAC greffé sur Rupestris du lot	Brasil
EU038294	RS	*V.vinifera*/Prosecco Tondo	Brasil
DQ526452	Ch-80	*V.vinifera*/Cabernet Sauvignon	Chile
AY371008	B19b	*V.vinifera*/Chardonnay	France
AY370941	A2b	*V.vinifera*/Chardonnay	France
AY371027	34d	*V.vinifera*/Chardonnay	France
AY370998	B10a	*V.vinifera*/Chardonnay	France
AY370975	A30f	*V.vinifera*/Chardonnay	France
AY370942	A5a	*V.vinifera*/Chardonnay	France
KJ913810	H5	Unpublished	Iran
FJ513376	KH-11-10-1	Unpublished	Iran
DQ362926	SG16	*V.vinifera*/Sangiovese	Italy
DQ362933	LS2	Grasparossa	Italy
DQ362927	MS43	*V.rupestris*/ St. George/Moscato	Italy
DQ362928	NE166	*V.rupestris*/St. George/Nebbiolo	Italy

Table 3. Newly designed primer used in this study

Primer	Squence	Location	Size
GT 1076	5'-CCAAGGATTGCCAGGCA-3'	1076-1092	750pb
GT 1826	5'-TCCATAGTGGTCCCGTTCC-3'	1806-1826	

3- Results

3-1 Serological results

The presence of GFLV and ArMVwas confirmed by Das-ELISA in 31% of samples from three vineyards located in two cultivars: Carignon and Muscat d'Alexandrie. Results show that not all the symptomatic cultivars were positive for ELISA. Symptoms observed on the ELISA negative samples can be attributed to other grapevine viruses.

3-2 RT-PCR results

65 samples of leaves and phloems were selected for nucleic acid extraction were tested for the presence of GFLV using FL C3310 / FL H2999 pair. Analysis by agarose gel electrophoresis of the PCR products (Figure 1) revealed the presence of the expected band in a total of 20 samples, which corresponds to 30.77% of the total samples.

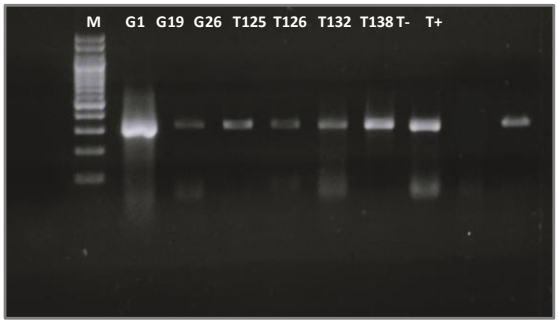

Figure 1. Agarose gel analysis of RT-PCR amplified products from some GFLV partial coat protein (312pb)(M: 100pb DNA Ladder (Bioron); G1, G19 and G26: positive samples from Grombalia ;T125,T126,T132 and T138: positive samples from Takelsa; T-: negative control; T+: positive control)

After annealing temperature using the new primers in the PCR, 57°C was the optimal for amplification. As well as the first pair of primer, we obtained 20 amplicon at the attended size (Figure 2) among them had not been amplified with the first pair of primers showed high intensity bands at a size of 750 bp

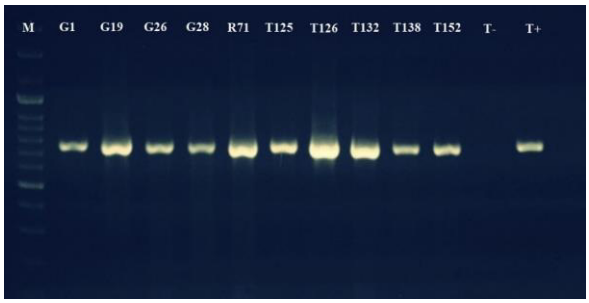

Figure2. Agarose gel analysis of RT-PCR amplified products from some GFLV partial coat protein (750pb) (M: 100pb DNA Ladder (Bioron); G1, G19, G26 and G28: positive samples from Grombalia ; R71: positif sample from Rafraf ;T125,T126,T132 T138 and T152: positive samples from Takelsa; T-: negative control; T+: positive control)

3-3 Genetic variability among GFLV isolates

11 local isolates obtained from three vineyards were selected for sequencing (Table4). Isolates were identified with"G"," R" and "T" correspond to the vineyard region: Grombalia, Rafraf and Takelsa respectively.

Table 4. The results of detection of isolates from different regions in Tunisia. a= amplidication of 312pb; b= amplification of 750pb; D= Deformation with acute dentations, LY= Leaf Yellowing, SI= Short internodes, Fasc= Fasciations, DN= Double nods, Fl= Shoot flattening

Isolate	Cultivar	RT-PCR[a]	RT-PCR[b]	Das-ELISA[c]	Das-ELISA[d]	Symptom
G1	Carignon	+	+	+	-	D
G19	Carignon	+	+	+	-	LY
G26	Carignon	+	+	+	-	LY
G28	Carignon	+	+	+	-	D
R71	Muscat d'Al.	-	+	-	-	SI+LY
T125	Carignon	+	+	-	-	D+ Fasc
T126	Carignon	+	+	-	-	SI+LY
T132	Carignon	+	+	+	-	App
T138	Carignon	+	+	-	-	Fl+SI+DN
T152	Carignon	+	+	-	-	Fl+D+SI
T160	Carignon	+	+	-	-	Fl+SI

Nucleotide sequences obtained ranged from 644 to 749 nucleotides. These sequences were aligned with ClusalW implemented into BioEdit 7.2.5 covering an area of 645pb (Figure3).

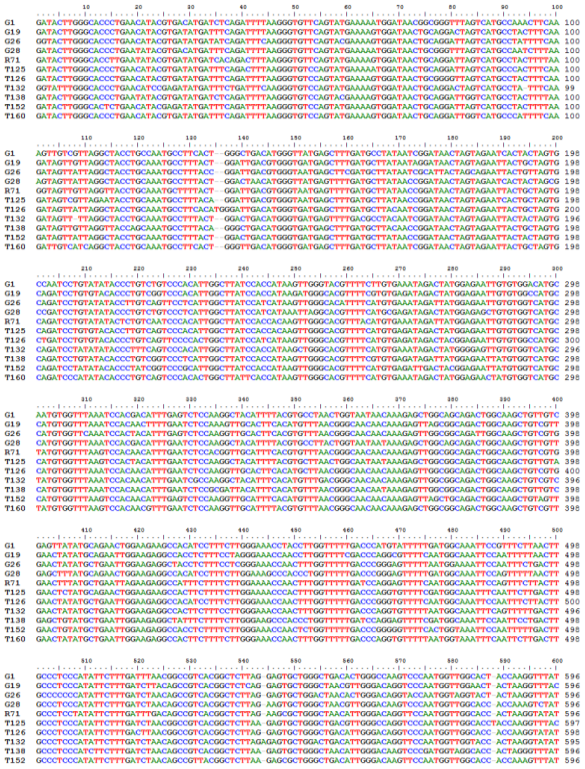

Figure3. Multiple alignment analysis of obtained partial sequences of $2C^{CP}$ GFLV

Despite the similarity observed among the isolates, the alignment show differences along the nucleotide sequence. Pairwise analysis was applied. All sequences provided 84.6% to 91.9% of identity (Table 5).

Table 5. Nucleotide sequence comparison between $2C^{CP}$gene of GFLV isolates

i1	G19	G26	G28	R71	T125	T126	T132	T138	T152	T160
D	86,20	84,60	90,60	84,60	88,70	87,00	85,80	85,70	85,60	87,60
	ID	90,40	86,80	89,60	89,80	91,90	90,00	89,50	90,70	90,70
		ID	85,00	88,90	87,00	90,10	89,50	88,20	88,40	89,30
			ID	86,40	88,70	87,20	85,90	87,30	87,50	86,70
				ID	88,60	89,50	89,00	89,50	89,20	89,30
					ID	89,40	87,50	89,00	88,90	90,80
						ID	90,30	89,00	90,90	91,40
							ID	87,80	90,00	91,20
								ID	90,40	89,30
									ID	91,50
										ID

Samples wich are positive for PCR and are negative for test ELISA reflects the high detection accuracy of PCR. Positive symptomatic samples of Das-ELISA with no amplification in RT-PCR can be explained by a low titer of GFLV in these samples to be detected by serological test. Pairwise analysis of GFLV isolates was conducted. It shows that the highest percent identity 91.9 % was observed between isolates from Takelsa (T126) and Grombalia (G19), followed by 89.6% between isolates from Grombalia (G19) and Rafraf (R71) and 89.3% between isolates form Takelsa (T160) and Rafraf (R71). Intra-regional analyses shows that within the region of Grombalia, the level of identity range from 90.4% between isolates G1 and G26 and 84.6 % between isolates G1 and G28. In Takelsa, T126 and T138 shows a level of identity of 89 %. However, T152 and T160 represent 92.5%.

3-4 Phylogenetic analyses

In order to understand the relationship between the Tunisianand theother GFLV isolates available at the GenBank (Table 6), a phylogenetic tree was established (Figure 4).

Table 6. List of GFLV isolates analyzed in this study

Origin	Accession	Isolates	References
	EU258681	IAC	
Brazil	EU258680	RUP	Unpublished
	EU038294	RS	
	DQ526452	Ch-80	
Chile	KC256954	Ch_FL1100	Unpublished
	KC256966	Ch_FL81	
	AY370941	A2b	Vigne *et al*., 2004a
	AY370942	A5a	Vigne *et al*., 2004a
France	AY370975	A30f	Vigne *et al*., 2004a
	AY371027	34d	Vigne *et al*., 2004a
	AY370998	B10a	Vigne *et al*., 2004a
	X16907.1	F13	Serghini *et al*., 1990
	AY997695	B5-7	Sokhandan Bashir *et al*., 2007
Iran	KJ913810	H5	Not published
	FJ513376	KH-11-10-1	Not published
	AY997697	Sh3-6	Sokhandan Bashir *et al*., 2007
Italy	DQ362933	LS2	
	DQ362926	SG16	Not published
	DQ362927	MS43	

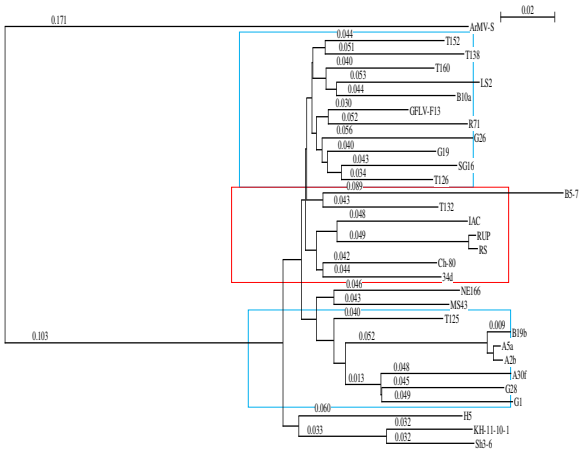

Figure4. Phylogenetic tree of GFLV isolates

The phylogenetic tree was generated using 31 CP sequences whose 20 were published in theGenBank (Table 6). As shown, isolates are divided in three main clusters. The first one regroup 6 Tunisian isolates (G19,G26,T126,T152,TT132,T160 and R71) wich are interspersed with twoFrench isolates (GFLV-F13andB10a) and two Italian isolates (SG16 and LS2). The second cluster contains 1 tunisian isolate T132 and Brazilian isolates (IAC, RUP and RS), Chilean isolate (CH-80) and French isolate (34d). The third one shows Tunisian isolates T125, G1 and G28 closely related to French isolates (A5a,A2b,A30f and B19b).

4- Discussion

GFLV was not detected in all symptomatic leaf samples, which can be explained by the low titer of the virus to be detected by Das-ELISA or may indicate that observed symptoms might be due to infection with others viruses.

A previous study analyzed 5 GFLV isolates in northern Tunisia by RT-PCR and partial sequencing of 312 pb long part of the CP gene using the specific primers FL C3310/ FL H2999 revealed a genetic variability ranged from 8% to 15.9% (Mrabet*et al.,* 2015). In order to investigate the genetic variation of GFLV in tunisiangrapevines , this study provides an RT-PCR assay using new specific primers designed to amplify a 750 pb region . Analyses of 11 CP sequences data reaveled genetic diversity ranged from 8.1% to 15.4% . Sequences comparisons showed that the primers generated in this study give the same rate of diversification comparing with the first couple of primers but they successfully increased the performance of the RT-PCR giving fragments showing high intensitieswith variable peaks in the graph . Although they indicate that within the same region, high nucleotide level of homology as well as high level of divergence can be observed. Therefore, no correlation between the level of divergence and geographic origin can be established.This result confirmed that the arrangement of these isolates is not correlated with their geographic origin.

The phylogenetic analysis showed an evolutionary relationship between Tunisian isolates and another previousely sequenced from different countries especially with france (F13) which can suppose that the origin of the viral infection in Tunisian vigneyads is the importation of plant material especially during the colonical period (Fattouch*et al*., 2005).

Based on other studies all over the world wich revealed an average of genetic diversity of approximatively 11% : Tunisia (Fattouch*et al*., 2005), Iran (Naraghi-Arani*et al*., 2001) and France (Vigne*et al.,* 2004b), this level is a common sight in RNA viruses (García-Arenal*et al.,* 2001) and the result of that GFLV as an RNA virus has no proofreading mechanism. Therefore, its replication is error prone and each isolate consists of a population of genetically related variants called "quasi-species" (García-Arenal*et al.,* 2001). This notion has been adapted to describe the evolution of viral populations. Generally, a viral population is subjected to fluctuations in time and space by mutation or recombination phenomenonwich is and evolutionary forces that intervene such as selection that favors the most suitable variants in an environment that promotes their fitness.

Data presented in this study provides new information about the genetic variability of Tunisian isolates which may help to improve new measures of control the sanitary status of planting material but a correlation between the genomic variation of viral sequences and the mechanism of their evolution must be established.

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