

Detection of a Region of the Coat Protein Gene of Grapevine Fanleaf Virus by RT-PCR in the Nematode Vector *Xiphinema index*

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ABSTRACT

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A region of the coat protein (CP) gene of grapevine fanleaf virus (GFLV) was detected by RT-PCR in the nematode vector *Xiphinema index*. The reverse transcription step was performed with total RNA extracted from viruliferous *X. index*. From the synthesized first cDNA strand, an 810-nucleotide fragment (nt 2515-3324 of GFLV-RNA2) was amplified by two 25-mer primers designed in the CP gene. Specific detection was successfully accomplished with samples of one or 10 viruliferous nematodes. Preliminary analysis and comparison of the 207-nt sequence of a stretch (nt 2549-2755 of GFLV-RNA2) of five different RT-PCR amplified fragments, obtained from 10 nematodes of a same sample in a diseased vineyard, indicated substantial variations in the GFLV CP gene. The deduced amino acid sequences showed important mutations essentially for two clones in four positions (residues 180, 188, 217, and 234 of the CP).

Additional keywords: nepovirus detection

Grapevine fanleaf virus (GFLV) is a nepovirus responsible for an economically important disease in vineyards all over the world (3,9,13). Plant prophylaxis has greatly limited the virus dissemination by vegetative propagation of infected cuttings and budwood. Transmission is now mainly by its vector, the nematode *Xiphinema index* Thorne & Allen. The GFLV genome is composed of two single-stranded, positive-sense, polyadenylated RNAs (RNA1 and RNA2) that carry a genome-linked protein (VPg) at their 5' ends (14). The capsid (CP) is composed of a single protein species of 56 kDa (18). RNA1 and RNA2 consist of 7,342 (15) and 3,774 nucleotides (nt) (20), respectively. A satellite RNA (1,114 nt) has also been identified (14). The sequence of RNA2 and the location of GFLV CP in the polyprotein were first determined in the French isolate F13 (20), which is able to encapsidate a satellite RNA, and were confirmed

in the Californian isolate from Davis (18), which lacks a satellite RNA.

A fast, reliable, and sensitive method for virus detection in its vector would be of particular interest. Immunosorbent electron microscopy allows virus detection in extracts of single nematodes (16) but has a limited interest for routine tests because of the complexity of the procedure. Until now, ELISA has been a reference method for routine virus diagnosis in grapevine plants (10,11). However, problems with ELISA detection occur in small nematode samples (2,5,6,21), and even with biotin-avidin amplification, the sensitivity of the method is not satisfactory in samples of fewer than 10 nematodes (7).

Virus detection within samples composed of small numbers of nematodes, and particularly from single individuals, would be useful for studies on epidemiology and virus-nematode interaction. Consequently, we used the reverse transcription-polymerase chain reaction (RT-PCR) method to detect a region of the GFLV CP gene in samples of one and 10 nematodes. This technique, combined with nucleic acid sequencing, enabled us to illustrate, in a preliminary

study, the variability of a GFLV CP region using nematodes all sampled in the same trench of a diseased vineyard.

MATERIALS AND METHODS

Viruliferous and virus-free nematode populations. A viruliferous nematode population was collected from a 14-yr-old vineyard (Chardonnay over 41 B rootstock) in Champagne (Mesnil-sur-Oger) France (8). All the viruliferous nematodes were sampled 40-70 cm deep in the same trench dug in the center of an area presenting marked fanleaf symptoms (3). Infectivity of each of the six plants located near the trench was confirmed by ELISA (10). The nematode population was reared in July 1992 on healthy grapes (*Vitis vinifera* L. 'Aramon' × *V. rupestris* Scheele, Ganzin No. 1 = AXR1) grown in containers in the greenhouse. After 1 yr, GFLV vectoring was confirmed on these plants by both symptoms and ELISA. Another viruliferous nematode population was collected in a vineyard near Montpellier, France, in 1990 and reared for 18 mo on fig (*Ficus carica* L.) to eliminate the virus. This virus-free population was then transferred to healthy AXR1 grapes. Tests on leaves (June 1992 and 1993) and roots (October 1992 and June 1993) by ELISA (10) confirmed the absence of GFLV in these later plants. Sampling and extraction of both populations were performed as described previously (8). Adult nematodes with a dark intestine (i.e., having already fed) were then hand-picked from the final water suspension and randomly separated into two groups. The first group of nematodes was submitted to ELISA. As reported by Esmenjaud et al (7), ELISA of increasing numbers of adults, ranging from one to 64 in multiples of two, confirmed the viruliferous and virus-free status of each population, respectively, and the approximate threshold of 10 nematodes for the

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detection of GFLV in *X. index*. The second group of nematodes was frozen at -80°C in sterile water before RT-PCR detection procedures.

Total RNA extraction from nematodes. For both viruliferous and virus-free nematodes, 10-nematode samples (i.e., the approximate ELISA detection threshold) were first processed. The samples were crushed and pulverized with a mortar and pestle previously cooled with liquid nitrogen. Individual *X. index* adults were crushed by a simplified procedure. Each nematode was placed in a 5- μl drop of sterile water on a coverslip and then ruptured by gentle pressure of a yellow, flat-tipped micropipette tip, which was sufficiently translucent to allow viewing the nematode and verifying rupture.

For both types of nematode samples (one or 10 nematodes), total RNA extraction was carried out with guanidinium HCl buffer (12) and Tris-equilibrated phenol-chloroform (pH 8.0), slightly modified for extraction from *X. index*. Nematode suspensions were added to 100 μl of guanidinium HCl buffer (8 M guanidine HCl, 20 mM MES [pH 7.0], 20 mM EDTA, 50 mM 2-mercaptoethanol) and mixed with phenol-chloroform (1:1, v/v). After centrifugation, the supernatant was precipi-

tated by adding 0.2 vol acetic acid (1 M) and 0.8 vol 95% ethanol. The pellet containing the total RNA was washed with 3 M sodium acetate (pH 5.2), centrifuged, and washed again with 70% ethanol. The total RNA pellet was then dissolved in 5 μl of sterile water and stored at -20°C .

First strand cDNA synthesis. Total RNA extract was incubated for 10 min at 65°C and quick-chilled on ice. First strand synthesis was carried out using an oligo d(T)₁₈ primer in total volume of 15 μl with 0.2 μg of primer, 13 mM DTT, and 200 units of murine reverse transcriptase and 1 μl of each of dNTP (10 mM). The RT reaction was then kept at 37°C for 1 hr.

PCR amplification. For PCR amplification assays, a 25-nt antisense primer (A3300; 5'-CCCACCAGCTTCGTGATGGTAACGC-3' complementary to nt 3300-3324) and a 25-nt sense primer (S2515; 5'-GGAAGAGGCCACTTCTTTCCTTGGG-3' corresponding to nt 2515-2539) were designed from the coat protein gene located in the RNA2 of the GFLV isolate F13 (20). The PCR amplification was carried out in a 50- μl final volume of *Taq* DNA buffer containing 1 μl of the heat-treated reverse transcriptase reaction, 8 μl of each dNTP (1.25 μM), 1 μl of *Taq* polymerase (0.5

unit), and 1 μl of each primer A3300 and S2515 (0.8 μM each) and overlaid with mineral oil. Temperature cycles were 5 min at 94°C , followed by 25 cycles of 60 sec at 94°C , 30 sec at 56°C , and 90 sec at 72°C . Amplified samples were subjected to electrophoresis in a 1% agarose gel, stained with 1 $\mu\text{g}/\text{ml}$ of ethidium bromide, and photographed.

The complete experiment from two distinct 10-nematode samples and from five distinct single-nematode samples was performed twice.

RESULTS

The two primers A3300 and S2515 from the coat protein gene located in the RNA2 were used to amplify an 810-nt fragment. As expected, an approximately 800-nt band was successfully amplified from samples of one and 10 viruliferous *X. index* adults. When first strand cDNA from virus-free adult nematodes was used, no amplification products were obtained (Fig. 1).

In order to confirm that the 800-nt amplified fragment corresponded to the region located between the two primers used (S2515 and A3300), fragments obtained from one of the 10-nematode samples were isolated from agarose gels and ligated to *Sma*I digested pUC19, according to standard protocols (17). Five recombinant clones were then sequenced by the dideoxy chain termination method (19).

The sequence corresponding to nt 2549-2755 of the GFLV RNA2 was analyzed. It represented 13.7% of the CP. In this region, one open reading frame with no stop codons was observed in all the sequenced clones. Nucleotide divergence between the five sequenced clones and the GFLV RNA2 reference isolate F13 varied from 6.7 to 13.5%, with an average of 9.5% (Table 1). The corresponding nucleotide sequence variations between the five clones and the GFLV RNA2 Davis isolate were 10.1-17.9%, with an average of 13.1% (Table 1). In these two cases, patterns of sequence deviation appeared to be restricted to nucleotide substitutions or small deletions and insertions. These substitutions were generally found in the third position of the codon.

Analysis of the 69 amino acid sequence corresponding to this region of the CP gene indicated substantial variations (Fig. 2). Amino acid sequence divergence between the five sequenced clones and the GFLV RNA2 reference isolate F13 (20) varied between 2.9 and 14.5%, with an average of 7.8% (Table 2). The corresponding amino acid sequence variations between the five clones and the GFLV RNA2 Davis isolate were 1.5 and 16.0%, with an average of 7.3% (Table 2). The degree of variation seemed to increase at particular sites along the nucleotide sequence, and four regions of frequent mutations were found in the sequenced

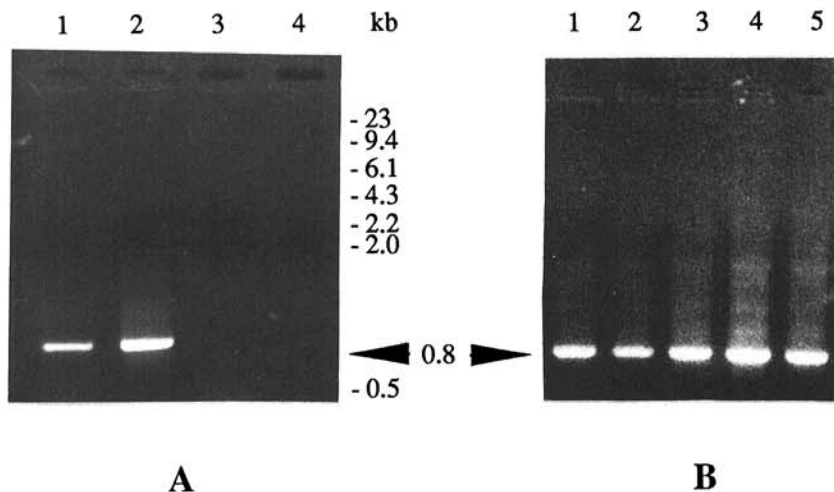


Fig. 1. Agarose gel analysis of a fragment of the grapevine fanleaf virus coat protein gene obtained by RT-PCR using total RNA isolated from samples of one and 10 *Xiphinema index* adults. (A) Ethidium bromide-stained products obtained from two samples of 10 viruliferous nematodes collected in the same field (lanes 1 and 2) and two samples of 10 virus-free nematodes (lanes 3 and 4). (B) Ethidium bromide-stained products obtained from single virus-infected nematodes (lanes 1-5). Molecular sizes of the ladder (*Hind*III digested lambda DNA) and amplified fragments are indicated.

Table 1. Nucleotide sequence divergences (percent) of the GFLV RNA2 (nt 2549-2755) of five sequenced clones compared with the reference GFLV isolate F13 sequenced by Serghini et al (20) and an isolate from Davis sequenced by Sanchez et al (18)

Isolate	Sequenced clones					Mean	GFLV Davis
	1.44	2.20	2.31	2.61	2.9		
GFLV F13	9.6 ^a	8.7	9.2	6.7	13.5	9.5	9.2
GFLV Davis	14.0	11.1	12.6	10.1	17.9	13.1	...

^aThe percent divergence calculation was performed with Mac Molly software in which insertion or deletion events were scored as a single change.

part of the coat protein (residues 180, 188, 217, and 234, considering GFLV F13 CP as the reference) (Fig. 2). Variations were generally restricted to single amino acid substitutions, except for clones 2.9 and 1.44; clone 2.9 exhibited blocks of two and three amino acid substitutions and clone 1.44 exhibited one block of four. Therefore, clones 1.44 and 2.9 appeared to be the most divergent variants, with 13.0 and 14.5% sequence divergence, respectively, from the reference isolate F13. They were also the most divergent variants from the Davis isolate (14.5 and 16.0%, respectively).

Amino acid substitutions resulted from single nucleotide changes in the codon, except for Cys to Leu in position 184 and for Pro to Ser in position 186, which required two nucleotide changes in their codons. Multivariant residues were found in four of the five sequenced clones at positions 180, 188, 217, and 234 (considering GFLV F13 CP as the reference), where amino acids Gln, Phe, Asp, and Arg were always substituted by His, Ser, Tyr, and Lys, respectively.

Furthermore, considering the degree of homology in the amino acid substitutions (based on chemical similarity of amino acid side chains), various situations were observed among the five clones. Clones 2.20 and 2.61 did not show any mutation involved in the structural and chemical modifications of the CP, whereas clone 2.31 showed only one non-conservative amino acid change (residue 188 of the CP), i.e., 1.4% divergence with the published sequences. This later substitution might be involved in some structural and chemical changes of the CP. Conversely, clones 1.44 and 2.9 showed three important amino acid changes (residues 188, 215, and 217 and residues 184, 187, and 217, respectively), i.e., 4.3% divergence with the published sequences. Therefore, nonconservative changes accounted for 33% (three of nine) and 30% (three of 10) of the substitutions in these two clones, respectively (Fig. 2).

DISCUSSION

Because grapevine fanleaf virus is present worldwide in vineyards with distinct *X. index* vector populations, studies on epidemiology and virus-nematode interactions must take into account the expected variability of both vector and virus (3,9,13). We used RT-PCR to amplify a region of the GFLV CP gene using total RNA extracted from single viruliferous *X. index* adults. Considering that the sensitivity of ELISA is not satisfactory with samples of fewer than 10 individuals (7), this method of GFLV detection from one individual is very promising for future studies of virus acquisition and retention in *X. index* and other potential *Xiphinema* vectors. For example, better knowledge of virus survival in the

nematode vector would provide useful data to break the ecological cycle of the virus-nematode complex, particularly between two vine crops. ELISA is a proven method for quantitative detection of GFLV in fields with high nematode numbers (7), and the RT-PCR method may be complementary by allowing the qualitative detection in single nematodes. Another more sensitive procedure for detection of GFLV in single nematodes would probably be immunocapture-PCR (4).

We also analyzed the potential of GFLV CP variability by sequencing five clones corresponding to RT-PCR amplification products from one sample containing 10 viruliferous *X. index*. Coat protein and its amino acid and nucleic acid sequence data should be most useful for further GFLV-nematode interaction studies. Our sequence analysis was done on 69 of 504 amino acid residues constituting the GFLV coat protein. Absence of stop codons in this region of the coat protein sequence indicated that the clones were not degenerated. Moreover, mean homology at the nucleic acid level was less important than at the protein level, as illustrated by clone 2.20, which exhibited only two amino acid changes

but presented 8.7% divergence at the nucleic acid level, compared with the GFLV reference isolate F13. The better conservation at the protein level could be attributed to the effect of the presumed selection pressure maintaining the coat protein gene fully active.

Amino acid sequences obtained with clones 2.20, 2.31, and 2.61 correlated very well with the published GFLV sequences (18). Conversely, unexpectedly high frequencies of amino acid substitutions were observed in clones 1.44 and 2.9. This cannot be explained by the synthesis and cloning methodologies using RT-PCR, since control experiments, with the same technique and reagents, on the sequences of three clones of the PCR-amplified acetylcholinesterase (*ace-1*) gene from *Caenorhabditis elegans* yielded an error rate lower than 0.1% (1). Therefore, we conclude that the appropriate region of CP gene was faithfully amplified.

As sequenced clones were obtained from a single RT-PCR using 10 viruliferous nematodes sampled in the same trench (100 × 40 cm, 40–70 cm deep), nucleotide changes should reflect variations within a same virus population. Although based on only 69 amino acids, it illustrates an intrapopulation vari-

			*		*	**	**	*		**
GFLV F 13	168	LVFDPGVFNG	KFQFLT	CPPI	FFDLTAVTAL	RSAGLTLGQV				
GFLV Davis		LVFDPGVFNG	KFQFLT	CPPI	FFDLTAVTAL	KSAGLTLGQV				
clone 1.44		LVFDPGVFNG	<u>KFH</u> FLT	CPPI	<u>S</u> FDLTAVTAL	<u>RS</u> I G LTLGQV				
clone 2.20		LVFDPGVFNG	KFQFLT	CPPI	FFDLTAVTAL	KSAGLTLGQV				
clone 2.31		LVFDPGVFNG	KFQFLT	CPPI	<u>S</u> FDLTAVTAL	KSAGLTLGQV				
clone 2.61		LVFDPGVFNG	KFQFLT	CPPI	FFDLTAVTAL	KSAGLTLGQV				
clone 2.9		LVFDP <u>G</u> L <u>F</u> NG	<u>KFH</u> <u>F</u> <u>E</u> <u>N</u> <u>L</u> <u>P</u> <u>S</u> <u>Y</u>		FFDLTAVTAL	<u>R</u> <u>T</u> <u>A</u> <u>G</u> LTLGQV				
GFLV F 13	208	PMVGTTKVVYN	LNSTLVSCVL	GMGGTVRGR	236					
GFLV Davis		PMVGTTTRVYN	LNSTLVSCVL	GMGGT I R GK						
clone 1.44		PMVGTT <u>TEG</u> LY	LNSTLVSCVL	<u>GME</u> GTI <u>KGR</u>						
clone 2.20		PMVGTTKVVYN	LNSTLVSCVL	GMGGTI RGR						
clone 2.31		PMVGTTKVVYN	LNSTLVSCVL	GMGGTI RGR						
clone 2.61		PMVGTTKVVYN	LNSTLVSCVL	GMGGTI <u>KGR</u>						
clone 2.9		PMVGTTKVVY <u>Y</u>	LNS <u>A</u> L <u>V</u> SCVL	GMGGTI RGR						

Fig. 2. Amino acid alignment of a fragment of the grapevine fanleaf virus (GFLV) coat protein from isolate F13 (EMBL accession X16907) sequenced by Serghini et al (20) and isolate obtained from Davis (EMBL accession X60775) sequenced by Sanchez et al (18) and five clones obtained by RT-PCR using total RNA from viruliferous nematodes. The first and last amino acids correspond to residues 168 and 236 of GFLV F13 coat protein. Each amino acid is represented by the standard single letter code. Amino acid changes in the sequenced clones are underlined. Asterisks indicate variable amino acid residues, and arrows indicate mutations implicated in potential structural and chemical changes of the coat protein.

Table 2. Amino acid sequence divergences (percent) of the GFLV RNA2 of five sequenced clones (amino acids 168–236) compared with the reference GFLV isolate F13 sequenced by Serghini et al (20) and an isolate from Davis sequenced by Sanchez et al (18)

Isolate	Sequenced clones					Mean	GFLV Davis
	1.44	2.20	2.31	2.61	2.9		
GFLV F13	13.0*	2.9	4.4	4.4	14.5	7.8	4.3
GFLV Davis	14.5	1.5	1.5	2.9	16.0	7.3	...

*The percent divergence calculation was performed with Mac Molly software in which insertion or deletion events were scored as a single change.

ability higher than that between the two described isolates F13 and Davis from France and the United States, respectively. However, one cannot exclude the possibilities that the variability observed in these sequenced regions may not reflect the overall variability of the GFLV coat protein and that amino acid variations may be lower in other regions. Since the CP is directly involved in the virus-nematode interaction (22), the knowledge of the intrapopulation variability of the whole CP sequence should be of particular interest for further studies on the putative role of the different mutations observed in the recognition between the virus and the nematode *X. index*.

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