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Phylogeny and genetic recombination of *Grapevine fanleaf virus* isolates from naturally infected vineyards in Tunisia

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Summary. The genetic diversity of *Grapevine fanleaf virus* (GFLV) was investigated in 11 isolates recovered from naturally infected vineyards located in the Cap Bon and Tunis regions. Virions were collected by immunocapture, and a 321 bp fragment containing part of the viral coat protein gene (CP) was amplified by RT-PCR. Amplicons were cloned into a pMos*Blue* vector and sequenced. The evolutionary historical relationships were determined by constructing a dendrogram using Maximum-parsimony (MP), Maximum-Likelihood (ML) and Neighbor-Joining (NJ), all of which gave similar results. Besides phylogeny, recombination also explains a considerable amount of genetic diversity in natural populations. Recombinational events were detected by a variety of bioinformatic programs. Different recombination breakpoints were determined with a particularly strong signal located downstream nt 272 of the amplicon (the position in the CP gene as determined by the RECCO algorithm is: 456–467). Interestingly, it was found that recombination is widespread in the CP gene even though only a short part of the CP cistron was analyzed. As a result of this analysis, it appears that recombination may explain in part the great number of GFLV isolates described worldwide. Apart from recombination, reassortment is still an open question among many others, and may represent another way to explore the genetic diversity of GFLV.

Key words: GFLV, sequence, evolutionary history, recombination.

Introduction

Grapevine fanleaf is the oldest known virus disease of grape (*Vitis vinifera*) (Martelli, 1986). It is thought that *Grapevine fanleaf virus* (GFLV) has coexisted with grapes since their earliest cultivation and has spread with the vegetatively propagated crop (Hewitt, 1970). In Tunisia, the disease is extremely widespread throughout the country, and is one of the major sources of loss for the Tunisian grapevine industry (Boulila *et al*., 1991).

Grapevine fanleaf virus (GFLV, genus *Nepovirus*, family *Comoviridae*) induces significant yield reduction and lowering of the quality of grapevine fruit and must, as well as vine degeneration. It causes malformation to the leaves, shoots and fruits, whereas some strains cause yellow discoloration of the leaves. Berry set on infected vines is reduced. Yield loss of up to 80% (Martelli and Savino, 1988) has been reported, in addition to lower quality and a reduction in vineyard longevity. Damage and malformation vary depending on the *vitis* species (and variety) and the virus isolate (Walter, 1998).

The GFLV virions contain a bipartite genome made up of positive-sense single stranded RNA, which carries a genome-linked protein (VPg) at its 5' end and a poly(A) sequence at its 3' end. RNA1 (7344 nucleotides) is translated as a P1 polyprotein of 253 kDa, and RNA2 (3774 nucleotides) as a P2 polyprotein of 122 kDa. The coat protein (CP) of

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504 amino acids (56 kDa) is produced by proteolytic cleavage at an Arg/Gly site in the C-terminal part of P2 (Pinck *et al*., 1991; Ritzenthaler *et al*., 1991). It should be noted that a satellite RNA, with only very limited sequence homology with genomic RNA, was found for GFLV F13 isolate (Pinck *et al*., 1988; Fuchs *et al*., 1989).

A wide range of symptoms are known to be caused by GFLV, depending primarily on the viral strain. GFLV includes distorting and chromogenous strains that cannot be distinguished serologically, morphologically or biologically (by host range response or vector transmission) from one another (Martelli, 1986).

The variation in GFLV symptomatology from fanleaf to yellow mosaic may have a genetic component (Naraghi-Arani *et al*., 2001). The level of genomic variation, as with many other plant viruses, suggests that the GFLV genomes consist of a genetically diverse collection of mutants, the dominant members of which vary during shifts between successive host varieties, in the manner of quasispecies which is a result of highly error-prone replication mechanisms (Roossinck, 1997).

Plant viruses frequently exploit recombination and reassortment as driving forces in evolution, and, occasionally, other mechanisms such as gene duplication and overprinting. The amount of variation found in different species of plant viruses is remarkably different, even though there is no evidence that the mutation rate varies (Roossinck, 1997). Recombination, defined as the exchange of genetic information between two nucleotide sequences, reshuffles existing variations and even creates new variants (Posada and Crandall, 2001).

The genetic diversity of GFLV found in Tunisian vineyards has been examined by Fattouch *et al*. (2005a,b). However, these authors did not discuss the occurrence of recombinational events in the genome of GFLV based on the Tunisian isolates.

It is nearly impossible to eradicate the virus from vineyards that have been planted with infected vines and that are infested with the dagger vector *Xiphinema index* (Andret-Link *et al*., 2004). The virus is spread both by infected propagating material and by vector feeding (Demangeat *et al*., 2004).

The aim of the present paper was to provide further information on the genomic variability, the evolutionary history and the genetic recombination of the GFLV, which is widespread in Tunisia.

Materials and methods

Virus source

Surveys were carried out in early spring 2005, and samples were collected from three economically important grapevine varieties: Carignan, Muscat and Grenache all growing in the Cap Bon and Tunis districts. Twenty-five accessions were collected. Each sample consisted of 25 shoots (collected from several vines) bearing leaves with virus symptoms and taken from the four compass points as well as from the internal part of the vine.

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

A single-step IC-RT-PCR was done as reported earlier (Wetzel *et al*., 1992).

Sample preparation. Applying PCR technology to *Vitis* usually presents problems because of the presence of high levels of phenolic compounds, polysaccharides, and other substances that make both nucleic acid extraction and virus detection difficult (Buzkan and Walker, 2004). To avoid such problems, the following extraction procedure was adopted (Rowhani *et al*., 1993): 1 g of fresh tissue was cut into small pieces, incubated in 10 ml of the extraction buffer (16.56 g K_2HPO_4; 4.1 g KH_2PO_4; 100 g sucrose; 1.5 g Bovine serum albumin [BSA] fraction V; 5 g of polyvinylpyrrolidone [PVP 40]; and 0.2% 2-mercaptoethanol l^{-1}). Mercaptoethanol was added last, and the pH was adjusted to 7.6). Grinding was carried out in individual plastic bags containing gauze (Bioreba, Reinach, Switzerland), and using a rolling grinder (Homex 6, Bioreba, Reinach, Switzerland). After grinding, the extracts were rapidly clarified by centrifugation (1000×*g* for 10 min, at 4°C).

IC-RT-PCR assay. The primers for GFLV were of the CP region located between nucleotides 184 and 505 with the sequences: 5'-CCA AAG TTG GTT TCC CAA GA-3' for the antisense primer, and 5'-ACC GGA TTG ACG TGG GTG AT-3' for the sense primer (Brandt *et al*., 1995).

One hundred microliters of clarified plant extract were subjected to immunocapture, which was performed directly in the tubes used for the reverse transcription PCR. The immunocapture tubes were coated using 2 μg of immunoglobulins per ml. The tubes were incubated overnight at 4°C in both steps. Following three washes with PBS-Tween buffer, the tubes were dried and further processed for amplifica-

tion. Fifty microliters of the RT-PCR mix (6 μl 0.3% Triton ×100, 5 μl 10× PCR buffer [100 mM Tris-HCl, pH 9, at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 1% Triton ×100; 2 mg ml^{-1} BSA]; 2.5 μl formamide 5%; 1 μl 10 mM dNTPs [2.5 mM dGTP; 2.5 mM dATP; 2.5 mM dCTP; 2.5 mM dTTP], 15 pmol of each of the two primers; 2.5 U AMV reverse transcriptase [Promega, Madison, WI, USA]; 1.25 U *Taq* DNA polymerase [Qbiogene, Montreuil, France], 40 U RNAsin [Qbiogene], and 32 μl sterile water) were added to each tube. After vortexing and quickly spinning down the droplets, the mix was overlaid with 50 μl mineral oil. The cycling scheme (MJ Research, Inc, Watertown, MA, USA) was the following: 30 min at 46°C (RT-reaction), 3 min at 95°C to denature the templates and the RTase, followed by 35 cycles of amplification: 30 s at 94°C (denaturation), 30 s at 50°C (annealing) and 45 s at 72°C (DNA synthesis). A final 7 min elongation step at 72°C was performed at the end of 35 cycles. Amplification products were analyzed by electrophoresis of 10 μl aliquots on 1.5% agarose gel, in 1× Tris-Borate-EDTA buffer (Sambrook *et al*., 1989). Bands were visualized by staining with ethidium bromide (5 μg ml^{-1}) and photographed using a UV transilluminator (ETX 20.M) at a wavelength of 312 nm and a Vilber Lourmat photo-print system (Model DP-001, FDC) (Vilber Lourmat, Marne-La-Vallee Cedex, France).

Cloning and sequencing of PCR products.

The cDNAs obtained by RT-PCR were cloned into the pMOSBlue plasmidic vector (Amersham, Buckinghamshire, UK). Briefly, the plasmid was linearized by digestion with EcoR V endonuclease, then ligation was performed using T4 DNA ligase (Amersham) according to manufacturer's instructions, followed by transformation into Escherichia coli DH5α. Ten clones obtained from each cDNA product were selected and the PCR amplified using the same conditions for the PCR as described above.

PCR products were subjected to nucleotide sequencing by dideoxy chain termination (Sanger *et al*., 1977), using the Big Dye Terminator Ready Reaction mix provided by Applied Biosystems (Foster City, CA, USA) in an automated sequencer (ABI-PRISM 377). Sequencing was done in both directions using the upstream and downstream universal primers T7 and U-19mer respectively. The resulting sequences were analyzed with CHROMAS 2.13 (Technelysium Pty. Ltd, Helenvale, Australia).

Computer-assisted sequence analysis

Databank searches for homologies to GFLV were undertaken using FASTA (Pearson and Lipman, 1988) and WU-BLAST 2, based on the Basic Local Alignment Search Tool algorithm (Altschul *et al*., 1990; 1994).

The nucleotide sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) with default parameters. The phylogenetic relationships were determined by three methods: neighbor-joining (NJ) with the CLUSTAL X (1.81) (Thompson *et al*., 1997) and MEGA 3.1 programs (Kumar *et al*., 2004); Maximum-parsimony (MP); and Maximum-likelihood (ML), using the PHYLO_WIN graphic tool (Galtier *et al*., 1996). Depending on the algorithm used, bootstrap analysis with 1000 replicates (NJ) or 500 replicates (MP and ML) was performed to assess the robustness of the branches.

Recombination events between diverged nucleotide sequences were explored with the PHYLPRO (Weiller, 1998), RDP2 (Martin *et al*., 2005) and RECCO programs (Maydt and Lengauer, 2006).

Results

GFLV PCR products

Immunocapture-RT-PCR successfully amplified the targeted genome portion of 11 of the 25 accessions collected. The size amplicon obtained (Fig. 1) ranged from 321 bp to 324 bp, as shown in the revealed sequences (Fig. 2). Based on the detection results, further investigations must be done to improve detection conditions, for instance, the Tris-HCl extraction buffer, recommended by M. Fuchs (personal communication), would give better results.

Nucleotide and deduced amino acid sequence analysis

To study the genetic diversity of GFLV isolates with special reference to their molecular variability, phylogenetic relationships, and the detection of recombination breakpoints, a comparative analysis of the nucleotide and amino acid sequences was done. The amplicons obtained from the isolate genome candidates were sequenced. Multiple alignments of the nucleotide (Fig. 2) and amino acid sequences were produced by CLUSTAL W (1.83) at two levels: the first pointed out the molecular variations between the Tunisian isolates, and it showed a di-

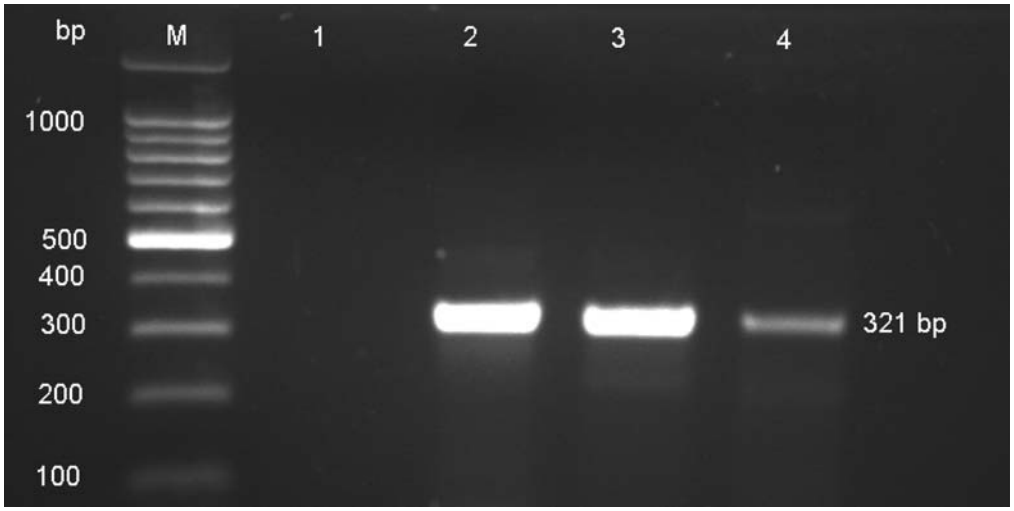

Fig. 1. Agarose gel of *Grapevine fanleaf virus* PCR products. M, Marker; lane 1, negative control; lane 2, positive control (provided by Bio-Rad, France); lane 3, CR1 isolate; lane 4, Muscat isolate. Bands correspond to amplicons having a size of 321 bp.

vergence of sequences ranging roughly from 86 to 98% and from 83 to 99% for the nucleotide and amino acid residues respectively. The second level included the Tunisian isolate sequences among several other sequences available at the GenBank/EMBL databases in order to establish the relationships of the Tunisian and also the foreign isolates.

The phylogenetic relationships between the GFLV isolates were inferred using NJ, MP and ML. The constructed trees basically gave a somewhat similar phylogenetic grouping. As shown in Fig. 3, isolates fell into 3 main clusters. Eight isolates (CR1, CR2, CR11, CR12, CR13, CR16, CR20, Grenache) were closely related to each other and to three previously described isolates collected from Tunisia by other workers and deposited in databanks under the accession numbers: AY525605, AY525606 and NC003623. The second cluster comprising CR-G1 and Muscat, had a quite close evolutionary history, and the third cluster, containing CR-M1, showed a wide difference in its genetic relationship with the other isolates.

Genetic recombination

To identify possible recombination events located within each amplicon, we used the PHYLPRO program which graphically displays the coherence of sequence relationships (phylogenetic correlation) over the entire length of a set of aligned homologous sequences. Recombination appears as areas of low phylogenetic correlation, visualized by sharp pointed downward peaks in the graph. In Fig. 4a, the genomic regions showed several putative recombination signals, suggesting possible recombination events that might have been blurred by mutations and other recombination events that had accumulated over time. Putative recombination breakpoints corresponded to positions 69, 85, 114, 132, 169, 202, and 297.

Since many of these sequences were possible recombinants, it could have been hypothesized that the possible recombination sites arose *in vitro* during RT-PCR; thus in order to avoid identification of false recombination events, we performed recombination analyses by applying the original RDP method (Martin and Rybicki, 2000), bootscanning (Salminen *et al*., 1995), the Maximum Chi Square (Maynard Smith, 1992; Posada and Crandall, 2001), Chimaera (Posada and Crandall, 2001), Sister Scanning (Gibbs *et al*., 2000), and Genconv (Padidam *et al*., 1999) as implemented in the RDP2 program (Martin *et al*., 2005).

The RDP and bootscan methods showed 1 potential event downstream of position nt 272; whereas Maxchi revealed 13 potential events, distributed mainly in 2 particular regions: the first 85 residues, and around the portion nt 180–296. Siscan detected 22 potential events dispatched on the whole portion corresponding to the area nt 35–305; while Chimaera found 4 potential events occurring in 3 distinct

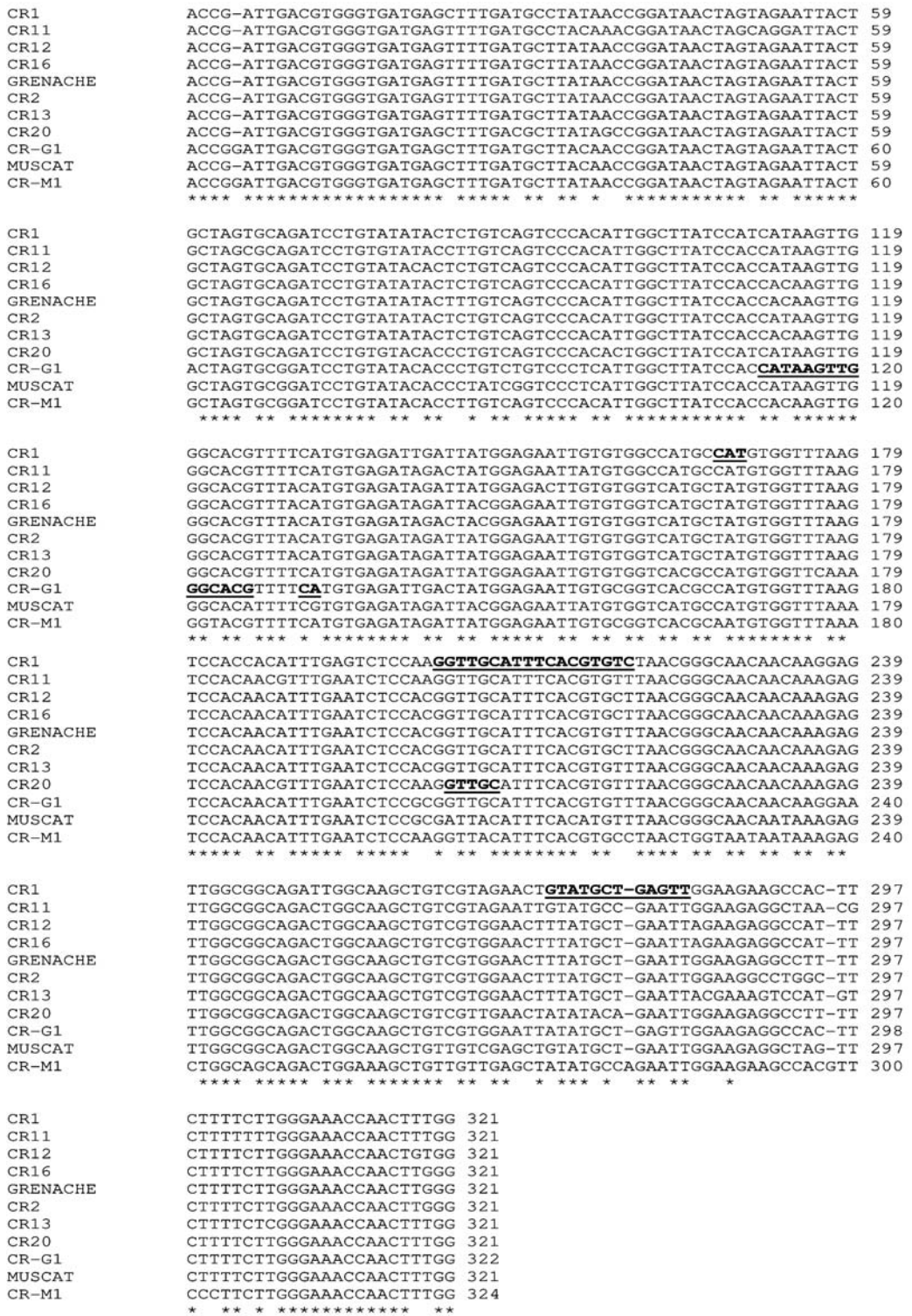

Fig. 2. Nucleotide sequence alignment of 11 *Grapevine fanleaf virus* isolates. Asterisks indicate the consensus sequence. Dashes indicate alignment optimization. Recombination site sequences revealed by the RECCO algorithm are shown in bold and underlined.

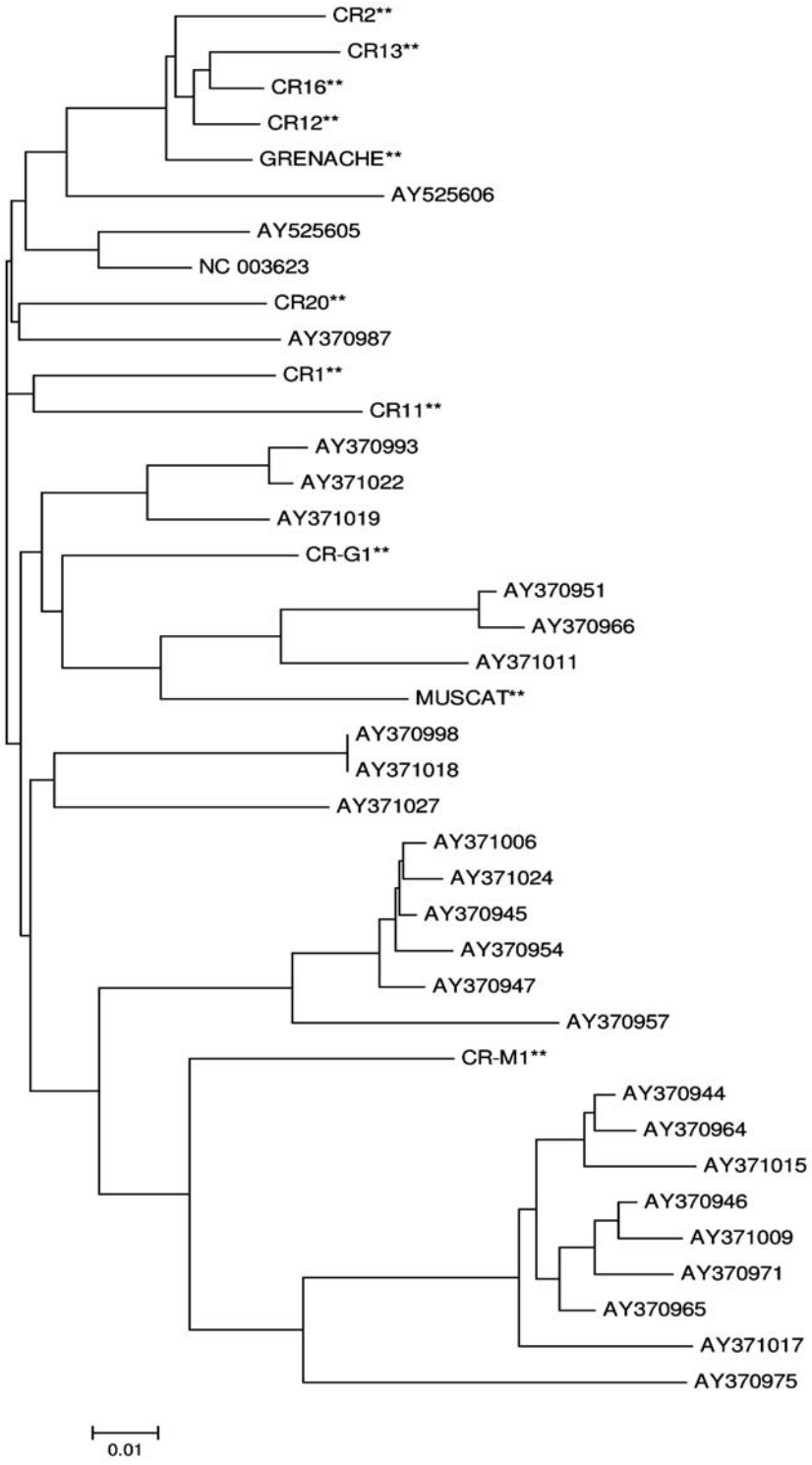

Fig. 3. Dendrogram depicting phylogenetic relationships among Tunisian isolates (with asterisks) compared to other isolates available in data banks. The tree was produced using the N.J. option of Mega 3.1 (Kumar *et al*., 2004). Bootstrap analysis of 1000 replicates was performed. The scale bar shows the number of substitutions per nucleotide.

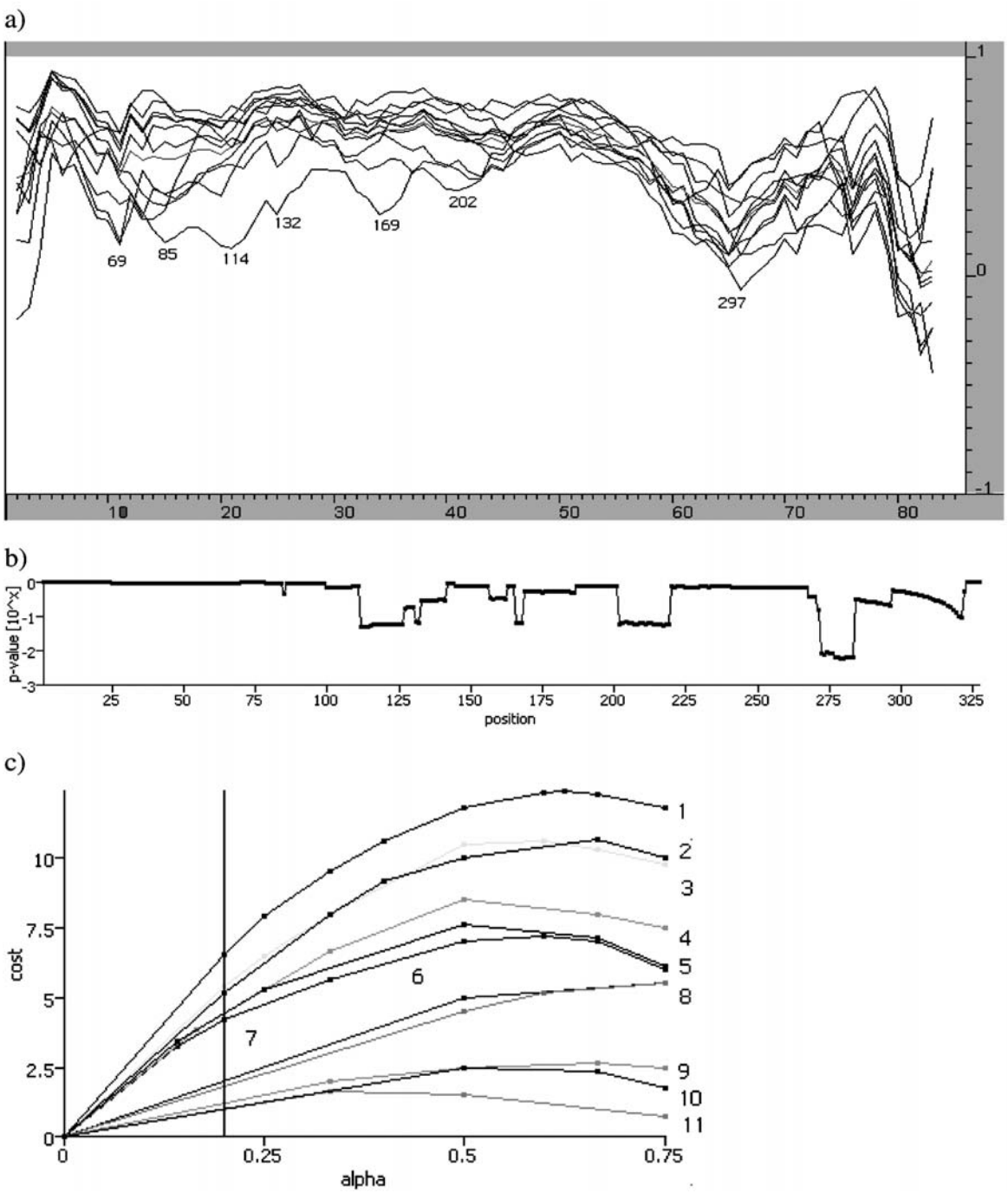

Fig. 4. (a) Graph of the phylogenetic correlations in the genomic sequences of eleven Tunisian isolates detected by the PHYLPRO method, which graphically displays the coherence of sequence relationships. Phylogenetic correlation was obtained at each variable site from pairwise distance analysis of all aligned sequences, with a fixed window of 40 bp. Numbers under low phylogenetic correlation areas (recombination signals) indicate nucleotide sequence positions; (b) Graph displaying breakpoint recombinations in the dataset detected by the RECCO method using cost optimization; (c) the parametric cost curves for each sequence (1, CR-M1; 2, Muscat; 3, CR11; 4, CR20; 5, CR-G1; 6, CR1; 7, CR2; 8, CR12; 9, CR13; 10, Grenache; 11, CR16). Parameter α controls the ambiguity between mutation and recombination. The current setting for α is shown as a vertical black line.

zones: around nt 30–170, nt 180–270 and downstream of position nt 272. In contrast, no potential event was detected with the Genconv method. The RECCO algorithm, described by Maydt and Lengauer (2006) as a fast, simple and sensitive method for detecting recombination in a set of sequences and locating putative recombination breakpoints that is based on cost minimization, was also used. This method has only two tunable parameters, recombination and mutation cost. In practice the only parameter considered is α, representing the cost of mutation relative to recombination. As shown in Fig. 4c, when α changes from 0 to 1, the cost of mutation weighted by α increases, and the cost for recombination weighted by 1-α decreases. In Fig. 4b, the p-value for recombination in the dataset is shown if the recombination event was the strongest in the whole data. The analysis yielded 6 recombination signals. Three recombination sites within the isolate CR1 sequence were detected: the first extended from nt 166 to nt 168 residue, whereas the second started at nt 202 and ended at nt 219, and the third was located between nt 272 and nt 284. The recombination breakpoints for CR-G1 were detected twice: from nt 112 to nt 126, and from nt 131 to nt 132. In contrast, only one recombination site was detected in the CR20 sequence extending from nt 203 to nt 207 (Fig. 2). Interestingly, based on the PHYLPRO and RECCO profiles (Fig. 4a, b), at least 4 out of 6 recombination sites were comparable. The RDP2 method, however, kept the putative recombination areas noticeably vague. All the methods used clearly showed a common recombination site located downstream of nt 272 of the amplicon, which corresponds to the position 456–467 in the CP gene as revealed by the RECCO algorithm.

Discussion

RNA viruses have a strong potential for genetic variation due to their error-prone RNA replication, large populations, and short replication times. As well as mutation, recombination is generally thought to be an important source of variability and an efficient tool to repair viral RNA genomes. Recombination events have undoubtedly contributed to the evolution of several plant viruses, and may have played an important role in virus adaptation (Simon and Bujarski, 1994; Lai, 1995; Nagy and Simon, 1997; Worobey and Holmes, 1999). Recombination in natural populations has been reported for several plant viruses (Fraile *et al*., 1997; Bousalem *et al*., 2000; Rubio *et al*., 2001; Ohshima *et al*., 2002; Glasa *et al*., 2004). For GFLV, each viral isolate is expected to consist of a population of genetically related variants, termed quasispecies (Garcia-Arenal *et al*., 2001). Some variant populations are reported to have an origin from mixed infection by two or more divergent GFLV isolates through successive infections by variants of different groups (Vigne *et al*., 2004a). In addition, the fact that heterogeneous populations of GFLV variants in a single vine (Naraghi-Arani *et al*., 2001) are likely to result from isolate mixing due to repeated transmissions on individual variants by viruliferous nematodes, is in agreement with the constant exposure of grapevines to *Xiphinema index* vectors for long periods of time (usually 30–40 years).

Recombination may occur in various genomic regions. The CP gene can be subjected to such genetic variation in numerous cases. Vigne *et al*. (2004b) previously reported the natural occurrence and widespread recombination of the nepovirus GFLV in the CP. This work confirmed the high variability of the CP gene that was detected using only a short part of that cistron (321 bp). Methods to detect breakpoint recombination showed consistencies with greater accuracy and sensitivity for RECCO, which in turn showed a limitation, as a long sequence (of a given recombination site) masked another shorter sequence, and both appeared as one in the dataset graph (e.g. CR1 masked CR20) (Fig. 4b).

Molecular evolutionary history and recombination studies of viruses are essential for drawing correlations between their genetic variation and geographical origins, as well as for the knowledge-based design of strategies for controlling viruses. For instance, in taking disease control measures, such as cross-protection or transgenic plant resistance, caution must be taken to avoid the introduction of exotic virus sequences that may recombine and give rise to new isolates with new biological properties.

It's noteworthy that besides recombination, genetic exchange can also be brought about by reassortment (i.e. pseudorecombination) (Pressing and Reaney, 1984). Roossinck (2002) and Bonnet *et al*. (2005) reported that reassortment may have led to the genetic diversity found among *Cucumber mosaic virus* strains and contributed to its enor-

mous evolutionary success. The increased genetic diversity afforded by reassortment may allow host range expansion and the recovery of strains bearing deleterious mutations.

One more open question in the evolution of GFLV is the role of genetic bottlenecks during transmission. The highly variable nature of GFLV could result in a dramatic loss of fitness if the bottlenecks are too narrow, a phenomenon known as Muller's ratchet (Chao, 1990; Chao *et al*., 1992). Finally, all the aspects mentioned above are still fertile areas of research to be explored by grapevine virology scientists.

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