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VIRAL AND VIROID DISEASES

Genetic variability of the movement and coat protein genes of *Grapevine fanleaf virus* isolates from Italy

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Abstract *Grapevine fanleaf virus* (GFLV), a nematode-transmitted virus belonging to the genus *Nepovirus*, is the major pathogen responsible for fanleaf degeneration, one of the most widespread and damaging viral diseases of grapevine. The virus is characterized by a genome constituted by two single positive sense RNAs (RNA1 and RNA2), coding for two polyproteins. Here we investigated the genetic variability of the movement and coat protein genes ($2B^{MP}$ and $2C^{CP}$) of Italian GFLV isolates by sequencing analyses. The presence of high molecular heterogeneity between our isolates suggests that GFLV comprises a family of sequence variants.

Keywords Coat protein · GFLV · Molecular diversity · Movement protein · Phylogenetic analyses · Sequence alignment

Fanleaf degeneration is one of the most widespread and damaging viral disease of grapevine, causing malformations of leaves and canes and foliage chlorotic discolorations. In Italy, *Grapevine fanleaf virus* (GFLV), a nematode-transmitted virus belonging to the genus *Nepovirus*, is the major pathogen responsible for the disease. GFLV has isometric particles of 30 nm diameter and contains two separate positive-strand RNA genome components: RNA1 and RNA2. Complete nucleotide sequencing of the GFLV's genome (isolate F-13) showed that RNA1 and RNA2 consist of 7,342 and 3,774 nucleotides (nt), excluding 3′ poly (A) sequences, which include single open-reading frames of 6,852 and 3,300 nt, respectively. RNA1 encodes five proteins, which are required for virus replication: 1A (proteinase cofactor), 1B (helicase), $1C^{VPg}$ (VPg), $1D^{Pro}$ (proteinase) and $1E^{Pol}$ (polymerase) (Margis et al. 1994; Ritzenthaler et al. 1991). RNA2 codes the homing protein $2A^{HP}$, the movement protein $2B^{MP}$ and the coat protein $2C^{CP}$ (Ritzenthaler et al. 2002).

The genetic diversity of GFLV has been assessed in several countries, and extensive variability has been reported in studies that were focused on the characterization of complete GFLV genome, full length RNA2 ORF, complete or partial $2C^{CP}$ and $2B^{MP}$ genes. This high level of genomic variability suggests that the GFLV genome may consist of a genetically diverse collection of mutants in the manner of a quasispecies (Naraghi-Arani et al. 2001).

In the present work, we molecularly characterized Italian GFLV isolates in their natural host plant, investigating the $2C^{CP}$ and $2B^{MP}$genes, in samples obtained from 16 GFLV-infected grapevines that had fanleaf degeneration symptoms and yellow mosaic (Table 1). Total RNA was extracted from 0.5 g of leaf sample from each grapevine plant using a QIAGEN RN easy plant extraction mini kit (Qiagen, Valencia, CA, USA), according to the procedure described by (MacKenzie et al. 1997). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using seven primer pairs: MP1-G1, M2-MP2, MP3–MP4, CP1–CP2, V-CPR1, FOR-CPR2, and CP3–CP4 (Table 2).

The amplified fragments from the 16 GFLV isolates were cloned using a pGEM-T Easy Vector Systems II (Promega, Madison, USA), and 1 clone per isolate was sequenced in both directions (Eurofins MWG Operon,

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Table 1 Italian *Grapevine fanleaf virus* isolates characterized by sequencing

Vitis vinifera cultivar/clone	Origin	Fanleaf severity	Accession
Sangiovese/SG1	Emilia-Romagna	Severe malformations	DQ362921
Sangiovese/SG4	Emilia-Romagna	Mild yellow mosaic	DQ362922
Sangiovese/SG10	Emilia-Romagna	Severe yellow mosaic	DQ362923
Sangiovese/SG11	Emilia-Romagna	Severe malformations	DQ362924
Sangiovese/SG12	Emilia-Romagna	Mild malformations	DQ362925
Sangiovese/SG16	Emilia-Romagna	Mild malformations	DQ362926
Lambrusco Salamino/LS2	Emilia-Romagna	Mild malformations	DQ362933
Lambrusco Grasparossa/LGR12	Emilia-Romagna	Mild malformations	DQ362934
Dolcetto/DO221	Piedmont	Severe malformations	DQ362920
Moscato/MS43	Piedmont	Mild malformations	DQ362927
Nebbiolo/NE166	Piedmont	Mild malformations	DQ362928
NebbioloNE83	Piedmont	Severe malformations	DQ362929
Nebbiolo/NE185	Piedmont	Mild malformations	DQ362930
Dolcetto/DO64	Piedmont	Severe malformations	DQ362931
Favorita/FA31	Piedmont	Mild malformations	DQ362932
Freisa/FR113	Piedmont	Mild malformations	DQ362935

Table 2 Primers used for molecular characterization of *Grapevine fanleaf virus* isolates

Primer	Sequence (5′–3′)[a]	Location in the RNA 2 genome (nt)[b]	Reference
MP1	CTG GGT AGG TTT GGT GGA CA	963–982	This work
M2	YTA GAY TTY AGG CTC AAT GG	1321–1340	Wetzel et al. (2001)
G1	TTT CCA AYG TRT TYG TCC C	1351–1369	Wetzel et al. (2001)
MP3	TAG CCA GGA GGA ACC AAG G	1614–1632	This work
MP2	TGC ATG GAR CCA ACG TGT TG	1726–1745	This work
CP1	GAT CCT CCC CAA CTT GAG GCT G	2003–2024	This work
MP4	TTC AGG GTG CC AAR TAT CT	2101–2120	This work
V	YGA TGC YTA TAA YCG GAT AAC TA	2259–2281	Naraghi-Arani et al. (2001)
CP2	AAA CGT GCC CAA CTT ATG	2344–2361	This work
FOR	GGA ACG GGA CCA CTA TGG AYT GG	2813–2835	This work
CPR1	TCT TCC ACA TAC ACC CCG GG	2854–2873	This work
CP3	TGG GTG GAT TTT TCT GAG TT	3097–3116	This work
CPR2	CAG GCA ATC ATY GCA GC	3208–3224	This work
CP4	TTT TAA AGT CAG ATA CCC TA	3562–3581	This work

[a] Y = C + T; R = A + G

[b] Relative to the GenBank accession number AY017 338 (Wetzel et al. 2001)

Ebersberg, Germany). Nucleotide sequence data were compiled and analysed using CLUSTAL W and MEGA version 5 programs (Tamura et al. 2007; Thompson et al. 1994).

Sequence alignment of the $2B^{MP}$ gene (1,044 nt) showed the presence of 214 parsimony informative sites and 141 singleton sites. Sequence analyses revealed that 286 amino acids (aa) of 384 aa of the movement protein were conserved among the 16 Italian GFLV isolates with an average number of amino acid differences per site (*p*-distance) of 0.033 (SE = 0.004). The majority of the variable amino acid sites seemed to be condensed in the C-terminal region of the protein. Our viral isolates shared nucleotide identities ranging from 79.8 % (isolate FR113 vs. LS2) to 98.7 % (NE185 vs. FA31) and aa identities ranging from 91.4 % (LS2 vs. NE166, NE185 and FR113) to 99.4 % (SG1 vs. NE83).The $2B^{MP}$ nucleotide and aa sequences of Italian isolates, compared with those of the GFLV isolates previously published in GenBank, revealed identities ranging from 74.9 % (FR113 vs. GHu from Hungary) to 92.9 % (NE83 vs. W18 from Germany) and from 88.5 % (SG4 and NE185 vs. GHu from Hungary) to 99.7 % (SG1 and NE83 vs. NW from Germany; A 17d from France; WAPN57, WAPN173, WAPN8133 and WAME1492 from the USA).

In the phylogenetic trees related to the $2B^{MP}$ nucleotide and amino acid sequences, the Italian GFLV isolates were generally found throughout the trees without particular subdivision in clusters, and all the isolates from Slovenia and Iran and some isolates from the USA and Germany formed distinct clades (Fig. 1 and data not shown).

Nucleotide sequence alignment of the $2C^{CP}$gene (1,512 nt) showed the presence of 349 parsimony

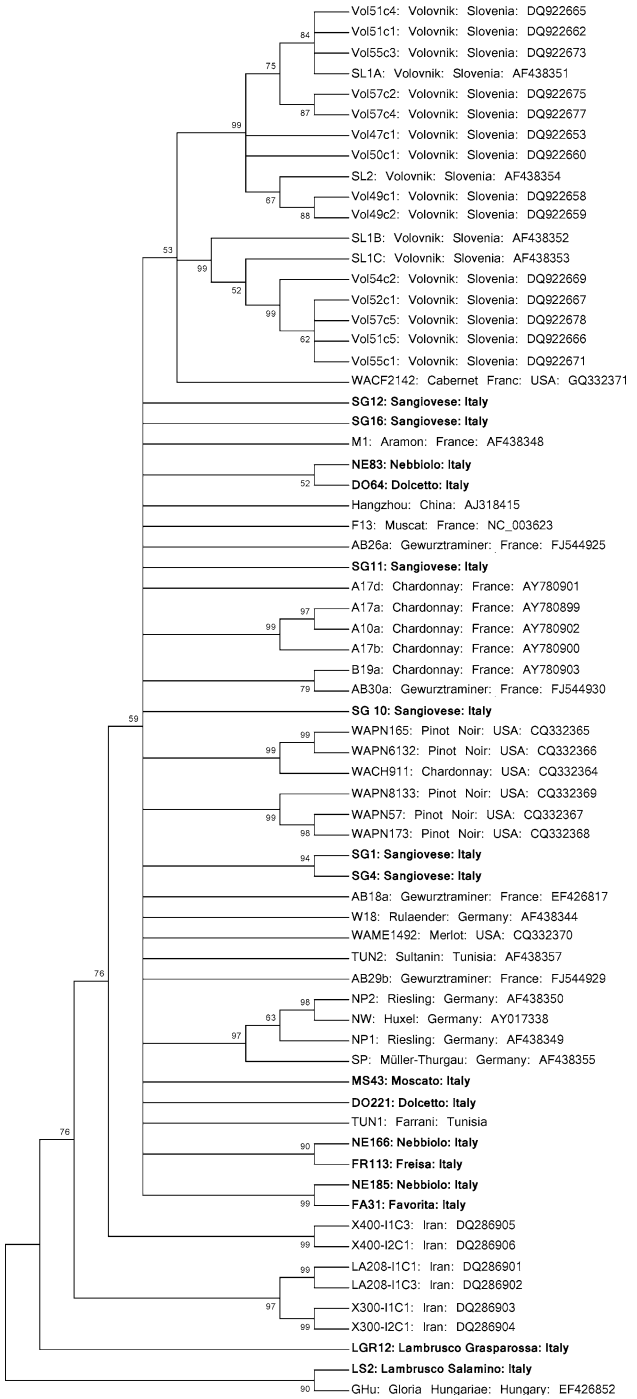

Fig. 1 Unrooted phylogenetic tree derived from the $2B^{MP}$ gene nucleotide sequences of 68 *Grapevine fanleaf virus* isolates and obtained by the neighbour-joining method. Host (*V. vinifera* cv.), geographical origin and GenBank accession number for each isolate are given. All Italian isolates are in *bold*. Bootstrap values were obtained from 1,000 replications, and bootstrap values <50 % were collapsed

informative sites and 173 singleton sites, and nucleotide diversity was distributed through the entire $2C^{CP}$ gene, without specific regions of high variability, confirming the data reported by (Naraghi-Arani et al. 2001) and Liebenberg et al. (2009). Amino acid sequence analysis of the $2C^{CP}$ encoded protein (504 aa) revealed 399 conserved residues among our 16 Italian GFLV isolates and an average number of amino acid differences per site (*p*-distance) of 0.049 (SE = 0.005). The Italian isolates shared nucleotide identities ranging from 85.2 % (FR113 vs. LGR12) to 99.4 % (NE185 vs. FA31) and aa identities between 93.7 % (DO221 vs. FR113; NE185 vs. FR113 and LGR12; FR113 vs. LS2 and LGR12) and 99.6 % (NE185 vs. FR113). Comparison between sequences of Italian isolates with those of the GFLV accessions published in the GenBank, showed nucleotide identities between 81.9 % (DO221 vs. S-4-2-1 from Iran) and 90.9 % (SG12 vs. Ch-80 from Chile and SG1 vs. F13 from France) and aa identity from 91.3 % (DO221 vs. KH-4-5-3 from Iran) to 97.6 % (SG12 vs. B3a from France), respectively.

The phylogenetic tree, based on the $2C^{CP}$ nucleotide and amino acid sequences, showed the Italian isolates clustered together with the majority of the published GFLV isolates in one branch and the Iranian isolates clearly most distant from all the others (Fig. 2 and data not shown).

This study has allowed us to characterize the $2B^{MP}$ and $2C^{CP}$ genes of the Italian GFLV isolates, confirming the genomic variability of this nepovirus. This high degree of genomic variability reveals that our GFLV isolates could represent a genetically diverse collection of closely related mutants subjected to continuous genetic variation, competition and selection as suggested by (Naraghi-Arani et al. 2001). Plant RNA viruses infecting perennial crops for long period share, in fact, potentially subjected to a greater genetic variation, and their replication process is error-prone, since no proof-reading mechanism is associated with RNA-dependent RNA polymerase.

Moreover, the absence of any significant relationship among geographical origin, sequence variability and grapevine cultivar within the Italian isolates analysed could also be explained by the separate introduction in Italy of different sequence variants by the extensive exchange of GFLV-infected grapevine germplasm and propagating material from distant viticultural areas of the world, supporting the hypothesis of (Meng et al. 2006).

On the other hand, this genetic variability could be explained by the interspecies recombination that occurs in RNA2 of grapevine-infecting nepoviruses. Due to the perennial nature of grapevines, different viruses can persist together in infected plants for many years, increasing the probability of recombinant isolate generation (Mekuria et al. 2009; Vigne et al. 2008).

In our analyses, we found no association between sequence variants and symptom severity, confirming that the $2B^{MP}$ and $2C^{CP}$ genes do not appear to be responsible for symptom expression, thus suggesting that viral

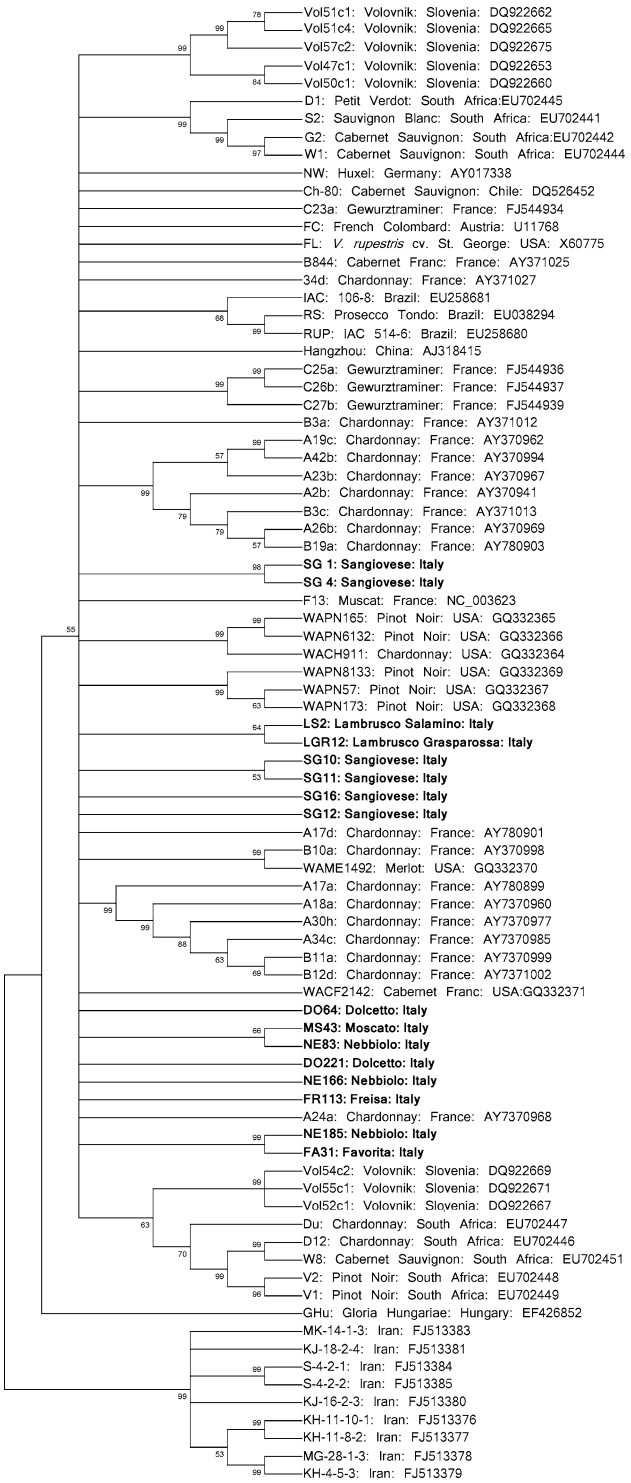

Fig. 2 Unrooted phylogenetic tree derived from the $2C^{CP}$ gene nucleotide sequences of 83 isolates of *Grapevine fanleaf virus* and obtained by the neighbour-joining method. Host (*V. vinifera* cv.), geographical origin and GenBank accession number for each isolate are given. All Italian isolates are indicated in *bold*. Bootstrap values were obtained from 1,000 replications, and bootstrap values <50 % were collapsed

determinants for symptomatology were probably within the RNA2 noncoding regions and/or on RNA1 (Liebenberg et al. 2009; Pompe-Novak M et al. 2007).

Up to now, several grapevine or rootstock GFLV-resistant transgenic lines have been produced (Gambino et al. 2005; Jardak-Jamoussi et al. 2009; Valat et al. 2006; Vigne E et al. 2004). The choice of GFLV gene sequence, on which the transgenic plant construct is based, represents the most important step to successfully produce resistant plants. For this region, the availability of data regarding evolution and genetic variability of the virus is essential.

The present study reports, for the first time, information about the genetic variability of Italian GFLV isolates. Our results clearly show a slightly higher variability in the $2B^{MP}$ gene when compared with the $2C^{CP}$ gene, confirming the importance of the CP region in the construction and use of resistant transgenic plants to control the virus. The intra- and interspecies recombination within the $2C^{CP}$ gene are, in fact, less prone to generate viable viral progeny than elsewhere in RNA2 genome (Mekuria et al. 2009; Vigne et al. 2008). However, better evaluation of the nucleotide variability of GFLV isolates in Italy as well as in other countries is essential to ensure durable transgenic resistance, even if the GFLV resistance mechanism is not yet well understood in CP-transgenic grapevine plants (Gambino et al. 2010; Vigne et al. 2009).

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