Analysis of Genetic Diversity and Phylogeny of Partial Coat Protein Domain in Czech and Italian GFLV Isolates

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Abstract

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The genetic diversity of *Grapevine fanleaf virus* (GFLV) was evaluated in 4 isolates sampled from naturally infected grapevines from South Moravia (Czech Republic) and 2 Italian isolates from Bari (Italy). Conserved regions within sequences in databases were found and new primers corresponding to these regions were designed and tested for RT-PCR amplification of the CP codifying region. After sequencing of obtained amplicons the similarity of isolates was analysed via alignments of sequences and by means of dendrograms.

Keywords: *Grapevine fanleaf virus*; grapevine; coat protein; sequence

Abbreviations: RT-PCR = reverse transcription polymerase chain reaction; CP = coat protein; MP = movement protein; 3'-NCR = non-coding region; VPg = genome-linked viral protein; nts = nucleotides

Grapevine fanleaf virus GFLV is the oldest known virus of grapevine (*Vitis vinifera* L.) (Martelli 1986). This member of the genus *Nepovirus* in the family *Secoviridae* (Sanfaçon *et al*. 2009) is the most widely spread. It causes a leaf degeneration disease and yield losses up to 80% can be incurred. Furthermore, the infection with GFLV reduces fruit quality and shortens the lifespan of vines (Martelli & Savino 1988). GFLV is naturally transmitted by the dagger nematode *Xiphinema index* (Esmenjaud *et al*. 1993) and experimentally by plant sap (Izadpanah *et al*. 2003).

The GFLV genome consists of two positive-sense single-stranded RNA molecules (RNA1, RNA2) encapsidated separately. Both genomic RNAs are covalently linked at their 5'-ends to a small viral protein (VPg) and are polyadenylated at 3'-ends. 3'-NCRs of RNA1 and RNA2 are identical in the framework of virus species (Le Gall *et al*. 1995).

At first the whole RNA1 and RNA2 molecules of GFLV were sequenced for strain F13 (Serghini *et al*. 1990; Ritzenthaler *et al.* 1991) and resultant lengths 7342 and 3774 nucleotides for RNA1 and RNA2 were established. The larger RNA1 encodes a polyprotein which is cleaved by RNA1-encoded viral protease into five products including proteinase and polymerase. RNA2 determines *in vitro* three products; N-terminal 2A protein implicated in the replication of RNA2, putative movement protein (2B protein) and coat protein (2C protein). From the point of view of the sequencing experiments, the complete RNA2 segment of GFLV German isolate NW (Wetzel *et al.* 2001) and five French isolates (Vigne *et al*. 2005) have also been determined. Furthermore, a lot of shorter sequences originating from different regions of RNA2 molecule were also published. Most frequently these are derived from GFLV coat protein (CP) gene, where for example the variability of the French isolates (Vigne *et al*. 2004, 2005) and the quasispecies nature of isolates (Naranghi-Arani *et al*. 2001) have been studied. Contrariwise, only

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few sequences originating from RNA1 molecule are currently available in databases.

The diversity of GFLV was determined in many regions over the world but today there are only two sequences derived from a GFLV isolate sampled in the Czech Republic. They are from the mild isolate HV5 from South Moravia (Czech Republic). These sequences were deposited in GenBank/NCBI under Acc. Nos. DQ386866 (the MP-CP) and AY821657 (the CP-NCR) (Komínek *et al*. 2006).

The aim of this study was to describe the sequence differences in the CP codifying region of Czech GFLV isolates and their comparison with Italian isolates.

MATERIALS AND METHODS

Virus. The isolates were detected by infected grapes from South Moravia (Czech Republic), specifically on the varieties KO1 (Kodrjanka), KML51 (Kišmiš Łučistyj), PN33 and PN35 (both Pamjati Negrula). The varieties have their origin in Moldova and they have been cultivated in South Moravia since 1990. Two Italian isolates 55TK and UR11 from Bari were also analysed (Digiaro *et al*. 2000). Total RNA was isolated from the grapevine phloem tissue scraped from dormant canes using Spectrum^{TM} Plant Total RNA Kit (Sigma, St. Louis, USA).

RT-PCR. Reaction for reverse transcription consists of water (HPLC purity), 0.25 µg random primer P(dN)6 (Roche, Indianapolis, USA) and 0.5 µg of total RNA in total volume 12.5 µl. This mix was denaturated at 95°C for 5 min at first. Subsequently 1× RT buffer (Fermentas, Bourlington, Canada), 0.2mM dNTPs (AppliChem, Darmstadt, Germany) and 200U reverse transcriptase M-MLV-RT (Fermentas, Bourlington, Canada) were added to final volume 20 µl. The time for reverse transcription was 60 minutes at 42°C. The reaction mix for PCR was prepared from water (HPLC purity), 1× DNA polymerase buffer, 0.2mM dNTPs (AppliChem, Darmstadt, Germany), 1U DNA polymerase (Finnzymes, Espoo, FInland) and 0.4mM self-designed primers CAE10 and EAC10 (Table 1). The primer hybridisation sites were designed at the conserved regions. These primers covered the CP codifying region encoding part from 2147 nts to 3416 nts of RNA2 strain. The temperature program for PCR was like in the experiments of Boulila (2007).

Sequencing and sequence analysis. The PCR products corresponding to the expected size were gel-purified using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and subjected to nucleotide sequencing by dideoxy chain termination (Sanger *et al*. 1977), using the BigDye® Terminator v3.1 (Applied Biosystems, Carlsbad, USA) kit. Separation of the fragments was done on an ABI-PRISM 310 genetic analyser (Applied Biosystems, Carlsbad, USA). Sequencing was done in both directions using all primers in Table 1. The obtained nucleotide sequences were analysed using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark) with default parameters. The phylogenetic relationships were determined within Maximum Likelihood Phylogeny analysis under assumption of the substitution model proposed by Jukes and Cantor (1969). Similarly like in Boulila (2007), for each resultant sequence the two most similar sequences from GenBank/NCBI were added to phylogenetic study (Figure 1).

RESULTS AND DISCUSSION

GFLV-derived PCR amplicons

RT-PCR successfully amplified the targeted genome portion of 6 isolates (KO1, KML51, PN33, PN35, 55TK, UR11). After gel electrophoresis the comparison with size standard shows that the size of obtained amplicons was as expected (approximately 1270 bp). Sequencing of PCR products was usually successful within the 935 nts long region (from 2240 nts to 3240 nts of RNA2). These 935 nts long sequences obtained in individual isolates

Table 1. Newly designed primers, the first two primers were used for PCR amplification (1270 bp), the rest of primers was used for sequencing. The first two primers are degenerate: Y = C + T and R = A + G

Primer	Length	Sequence	Location/Binding sites
CAE10	20	5'-GGTGTYCAGTATGAAAARTG-3'	RNA2/2147-2166
EAC10	22	5'-CTGCAAAATTCCCAAYCAACAA-3'	RNA2/3395-3416
SQCE10b	19	5'-ACAATAAAGAGTTGGCGGC-3'	RNA2/2460-2478
CAE-10-fwdseq	18	5'-ATGGAGAATTGTGTGGTC-3'	RNA2/2376-2393

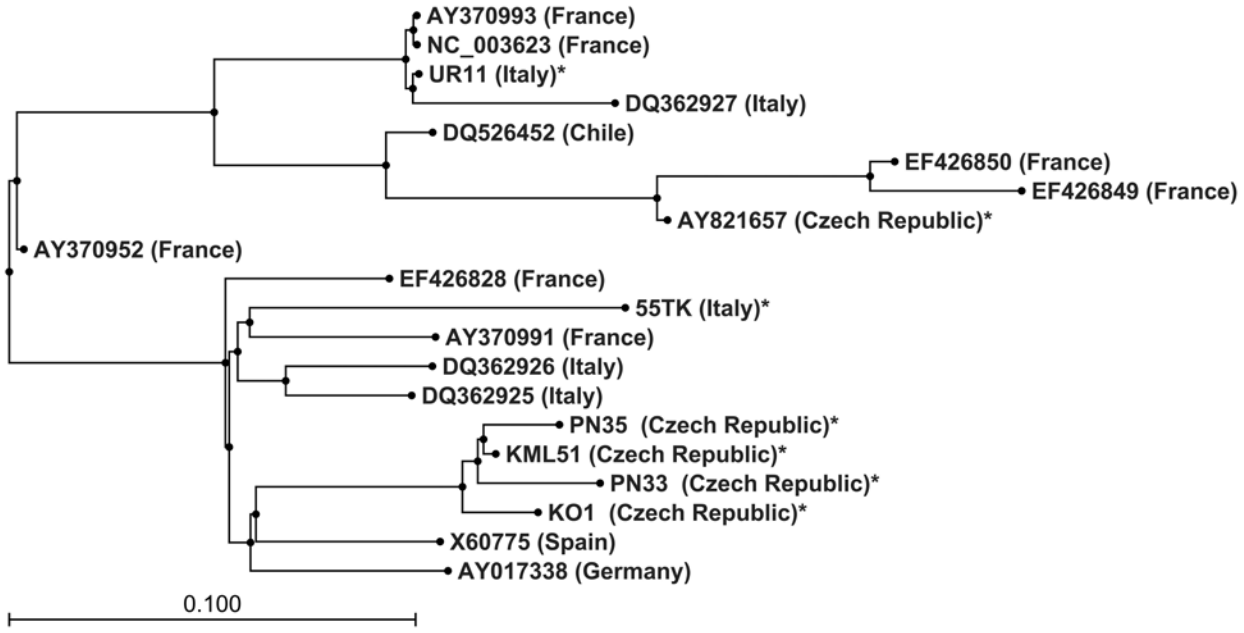

Figure 1. Phylogenetic tree of GFLV isolates. The isolates newly sequenced were labeled by stars in this figure. Scale bar represent a genetic distance of 0.100

were assembled to reference sequence NC_003623 (Serghini *et al.* 1990) and subsequently compared. The obtained sequences were deposited in GenBank/NCBI under Acc. Nos. GU053731 (KO1), GU062186 (KML51), GU053732 (PN33), GU053733 (PN35), GU053734 (55TK), GU053735 (UR11).

Nucleotide and deduced amino acid sequence analysis

To study the genetic diversity of GFLV isolates with special emphasis on their molecular variability and phylogenetic relationships, a comparative analysis of obtained sequences was done. The most variable regions at nucleotide and amino acid levels were detected by CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark) software. Generally, the similarity of sequences ranged from 83% to 86% and from 81% to 91% for the amino acid residues, respectively.

Phylogenetic study

The phylogenetic relationships between the GFLV isolates were inferred using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark) (Figure 1). Apparently, the obtained dendrogram divided whole group of estimated sequences into three main clusters where the isolate AY370952 (France) is the intermediate one. The results confirmed that arrangement of isolates within this dendrogram is not strictly based on their origin. The Italian isolate UR11 with reference sequence NC_003623, another Italian isolate DQ362927 and the French isolate AY370993 were included in the first cluster. The second cluster contains sequenced Italian isolate 55TK, two French isolates and two Italian isolates that all were the most similar to the isolate 55TK. That is why there is an interesting distance between the Italian isolates UR11 and 55TK. According to the facts mentioned above, the newly sequenced Czech isolates are quite unique because they created their own cluster different from the others. They proved relatively high degree of mutual similarity. This research could be the confirmation of the fact that all Czech isolates were obtained from grape cultivars introduced together from Moldova 30 years ago and the fact that the sampling was done in localities relatively close to each other (hundreds of meters). Another isolate HV5 (AY821657) from South Moravia (Czech Republic) sequenced and described by Komínek *et al.* (2006) is much different from the newly sequenced isolates in this study. The fact that the HV5 isolate is different supports the insights above.

Generally, our results confirmed the high variability of the CP gene that was detected within

the sequenced 935 bp long part of the cistron. The phylogenetic study shows evident similarity of the newly sequenced Czech isolates from South Moravia and their relatively high dissimilarity from the rest of analysed isolates including another previously sequenced South Moravian isolate HV5.

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