PARTIAL MOLECULAR CHARACTERIZATION OF A MILD ISOLATE OF GRAPEVINE FANLEAF VIRUS FROM SOUTH MORAVIA, CZECH REPUBLIC

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Summary. – An atypical mild isolate HV5 of Grapevine fanleaf virus (GFLV) was found in a South Moravian viticulture region in Czech Republic. Partial sequence of its RNA2 was determined and compared with available sequences of typical GFLV isolates. Two genomic regions, namely a 814 nt-long one spanning the movement protein (MP) gene and a 5'-part of the coat protein (CP) gene, and a 1426 nt-long one covering a part of the CP gene and the adjacent 3'-non-coding region (3'-NCR) were analyzed. Although no HV5-specific molecular features could be found in the two regions, marked differences were observed in the 3'-NCR. There was a 54 nt-long portion in which the sequence identity of some compared isolates was only 54.7%. Moreover, an unique one-nucleotide deletion occurred in the HV5 3'-NCR. These changes were also reflected in the predicted RNA secondary structure of this region. Particular biological behavior of GFLV HV5 isolate, namely a symptomless infection, could be related to the observed molecular differences.

Key words: Grapevine fanleaf virus; movement protein; coat protein; secondary RNA structure

Introduction

GFLV is a worldwide-distributed pathogen causing economically the most important disease of grapevines (Auger *et al*., 1992). Symptoms of GFLV infection on leaves may vary from "fanleaf" shape deformation to yellow mosaic and vein banding (Krake *et al*., 1999). The virus is transmitted by a longidorid nematode *Xiphinema index* (Andret-Link *et al*., 2004). A major way of long-distance dissemination is vegetative propagation or grafting of infected material.

The genome of GFLV (the species *Grapevine fanleaf virus*, the genus *Nepovirus*, the family *Comoviridae*) consists of two single-stranded RNA molecules encapsidated separately. Both genomic RNAs are covalently linked at their 5'-ends to a small viral protein (VPg) and polyadenylated at their 3'-ends. 3'-NCRs of the two genomic RNAs are identical for many nepoviruses (Le Gall *et al*., 1995).

The larger RNA1 (7.3 kb) encodes a polyprotein, which is cleaved by an RNA1-encoded viral protease into five products including the proteinase and a polymerase. RNA2 (3.8 kb) determines *in vitro* three products; the N-terminal 2A protein implicated in the replication of RNA2, the putative MP (2B protein), and CP (2C protein).

During a recent immunological survey in Czech Republic, GFLV was found to be present in cultivated grapevines (Komínek and Holleinová, 2003). Although most of GFLV-infected vines displayed typical fanleaf symptoms, one lacking obvious symptoms was recorded in South Moravia. In order to search for potential molecular differences, a mild GFLV isolate obtained from this plant was partially sequenced and the obtained sequences were compared to those of typical GFLV isolates so far available.

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Abbreviations: CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; GFLV = Grapevine fanleaf virus; MP = movement protein; 3'-NCR = 3'-non-coding region; Rep = replicase; VPg = genome-linked viral protein

Materials and Methods

Virus. The vine plant from which the HV5 isolate of GFLV was obtained was found in a vineyard near Horní Věstonice in a viticulture region in South Moravia. Contrary to other infected vines, this showed a mild mosaic but no typical fanleaf symptoms. The presence of GFLV was confirmed by double-antibody sandwich ELISA (DAS-ELISA) using commercial antisera (Agritest Valenzano, Italy). Cuttings from the original infected plant were collected and grown in greenhouse.

Total RNA was isolated from grapevine phloem tissue scrapped from dormant canes using RNeasy Plant Mini Kit (Qiagen) according to MacKenzie *et al*. (1997).

RT-PCR was carried out as described earlier (Komínek *et al*., 2005). Two regions of GFLV RNA2 were amplified (Fig. 1). For an 814 nts-long region (nt 1339–2152) encompassing two thirds of the 3'-part of the MP gene and a 5'-part of the CP gene the primers M2A (5'-YTRGATTTTAGGCTCAATGG-3', sense) and M4R (5'-GTDATCCACTTYTCATACTG-3', antisense) were used (Wetzel *et al*., 2002). An 1426 nts-long region (nt 2280–3706) spanning the CP gene and the 3'-NCR was amplified by a semi-nested PCR according to Naraghi-Arani *et al*. (2001). The first PCR was run with the primers NarAra rev (5'-ATTTGCATAA CAGTAG-3', antisense) and NarAra nestA (5'-TGATGCTTATA ATCGGATAACTA-3', sense), while in the second (nested) PCR the primers NarAra nestR (5'-CAAGGCAAGTGTGTCCA-3', antisense) and NarAra nestA were used.

Cloning, sequencing and sequence analysis. The PCR products corresponding to the expected fragments were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the pGEM-T Easy plasmid (Promega). Plasmids were propagated in *Escherichia coli* JM109 and isolated with the JETquick Plasmid Miniprep Spin Kit (Genomed). Clones were commercially sequenced using the MegaBACE™ 1000 DNA Analysis System (Amersham Biosciences). To determine one sequence, four clones were sequenced in both strands using universal pUC primers. The obtained sequences were analyzed using the MEGA Program version 3.1 (Kumar *et al*., 2004). Sequences of other GFLV isolates were retrieved from the GenBank (Benson *et al*., 2005; www.ncbi.nlm.nih.gov). RNA secondary structure was predicted through web interface Vienna RNA server using default parameters (Hofacker, 2003).

Results and Discussion

Contrary to common GFLV isolates, the infection of grapevine with HV5 was pronounced only by a very mild leaf mosaic; later in the season the reaction was symptomless. However, absence or mildness of symptoms was not due to lower concentration of the virus in plants. Virus titers estimated by a semi-quantitative DAS-ELISA showed no significant differences between HV5 and a typical fanleaf isolate (data not shown).

In order to check if such a biological behavior could reflect some difference at the molecular level, partial RNA2 sequence of HV5 was determined. Two regions were chosen: the MV gene with adjacent part of the CP gene (the MP-CP) and the CP gene with adjacent 3'-NCR (CP-NCR) (Fig. 1). The obtained sequences were deposited in GenBank under Acc. Nos. DQ386866 (the MP-CP) and AY821657 (the CP-NCR).

Fig. 1

Schematic diagram of the GFLV RNA2 genome

Two sequenced regions are grey-colored.

The 814 nts fragment containing no initiation or termination codon corresponded to 271 amino acids in MP. Multiple alignment showed a strict colinearity of the sequences of HV5 and 8 other isolates from France, Germany and China (NC_003623, AY780899-AY780903, AJ318415). HV5 shared with them a 87–91.1% identity at the nucleotide level and a 96.2–100% identity at the aminoacid level. Full aminoacid identity was observed between HV5 and the F13 isolate from France. Moreover, an STVR/G cleavage site between MP and CP was identical for all the isolates under comparison. The observed high level of conservation of MP among different isolates is not surprising. Belin *et al*. (1999) have proven that last 9 amino acids are necessary for a proper function of GFLV MP. As no particular amino acid differences could be found in the HV5 MP, this region is probably not responsible for the symptomatology of GFLV infection.

The 1426 nts fragment corresponded to 426 amino acids in CP. Similarly to other GFLV isolates, it contained a TAG stop codon in the positions 3560–3562. Comparison of HV5 with 24 other isolates showed an identity of 86–89.5% and 93.7–96.4% at the nucleotide and aminoacid level, respectively. Most of differences were located in the C-terminal part of CP. However, similarly to the 814 nts fragment, no particular aminoacid differences could be found in this part of the HV5 genome. Based on the CP sequence, the HV5 isolate was most closely related to the French isolates with Acc. Nos. NC_003623 and AY371011. Phylogenetic analysis showed no obvious geographical structuration of the GFLV isolates (Fig. 2).

Interestingly, greater differences among GFLV sequences were observed in the 3'-NCR. There was a 54 nts-long portion (nt 3585–3638), in which the identity between individual sequences dropped up to 54.7%. Moreover, a unique deletion of one nucleotide occurred in the HV5 3'-NCR at the position 3619 (Fig. 3).

To further highlight these differences, the predicted secondary structures of the 3'-NCR RNA regions of HV5

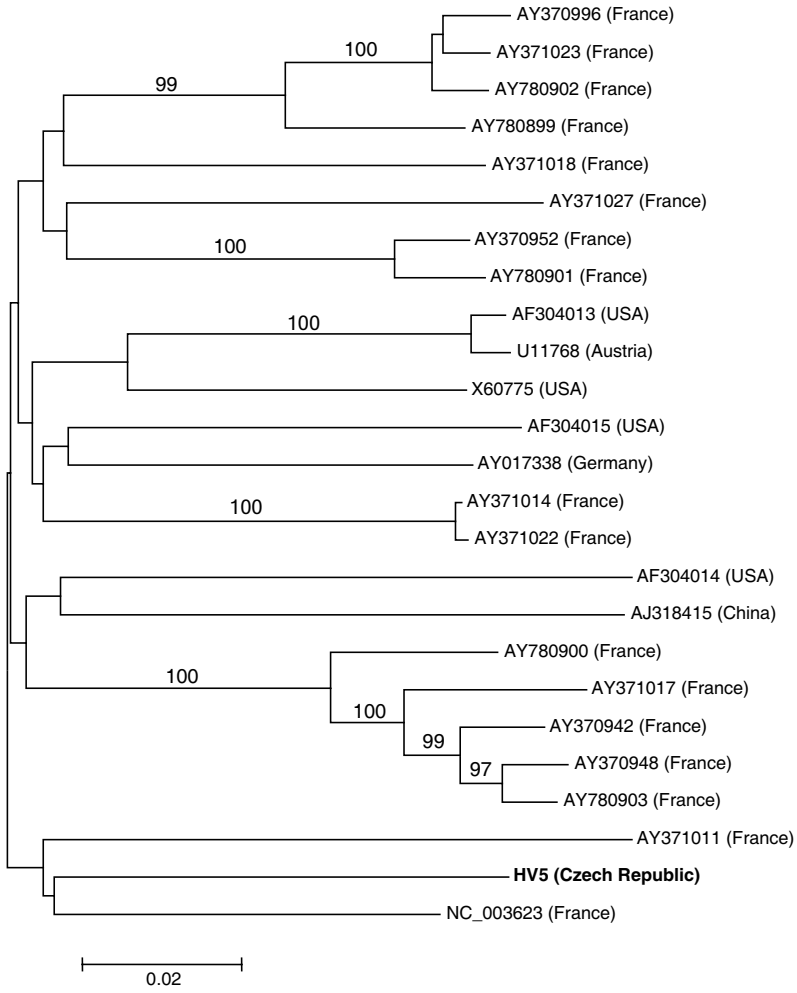

Fig. 2

Phylogenetic tree of GFLV isolates

Unrooted neighbor-joining phylogenetic tree, based on partial CP amino acid sequence. Bootstrap values higher than 60% are indicated at branch points.

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                3563
HV5             GGTATCTGACTTTAAAAGACCCAAATGTATATACGTATTTCGTTAATTGTGCGTGT-ATT
NC_003623       ......................G........T..G...T..C.G.A.CAT..A.T...
AF304013        ......................TG........T...A..T........CAT..A.TG..
AF304014        ......................GG....C...G..GC..TA...G..A..T...CTG..
AF304015        ......................G....C...T..G...T....G..A..T...CC...
AY017338        ......................GG....C......GC..T....GC.A..T....TT.C
X60775          ......................GCG........T...A..TA.......CAT....T...

HV5             TTGTGTTATGATTGCTTTTAACTTGTTTACTGCTTTAGTGTGTTTAGTTTCATGCTTTTA
NC_003623        ........A...TG.............T.C.....T.......................
AF304013         ........TG..TG.............................C...A.............
AF304014         ...A..G.AT..TGC..........C...C...............T.....C......G
AF304015         ........AG..TG..............C...............T.............
AY017338         ...A..G.A...TGC.............................T.............
X60775           ...A..G.AG..TGC.............................A.............

HV5             GTGGCGACAGTGTGTTGTTTGTCC 3706
NC_003623       ........................
AF304013        ........................
AF304014        .......T................
AF304015        ........................
AY017338        ........................
X60775          ........................
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Fig. 3

Multiple sequence alignment of the 3'-NCR of GFLV isolates

A highly variable 54 nts-long portion is highlighted. Only differing nucleotides are indicated. Nucleotide numbering according to the complete GFLV RNA2 sequence.

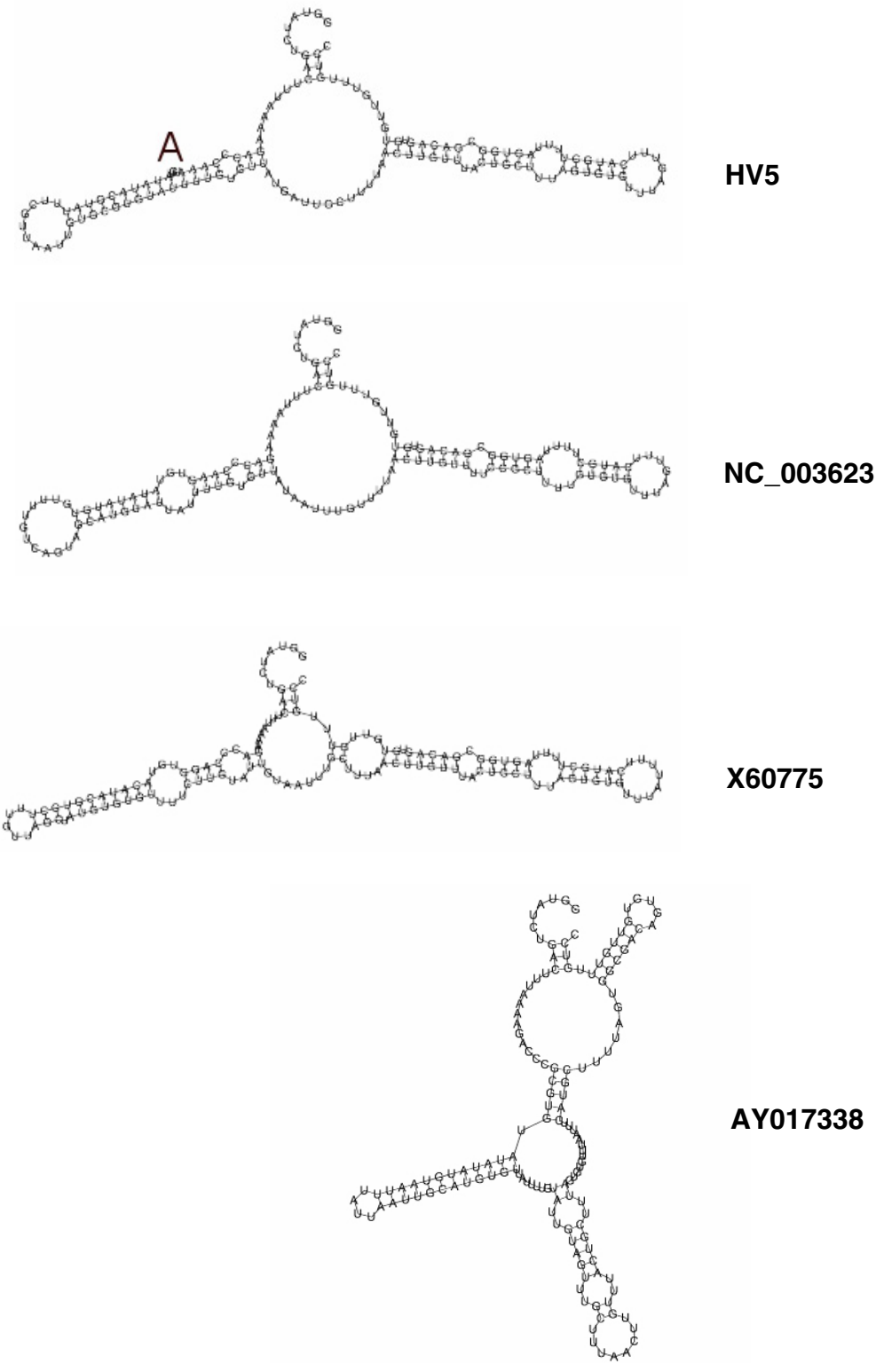

Fig. 4

The predicted secondary RNA structure of the 3'-NCR region of selected GFLV isolates

(A) indicates a missing loop in the secondary structure.

and three selected GFLV isolates (Acc. Nos. AY017338, NC_003623, and X60775) were computed. Typical "stem and loop" structures were observed for all the isolates (Fig. 4). However, the differences in the secondary RNA structure among GFLV isolates due to a nucleotide variability and one-nucleotide deletion could be noted. Particularly, there was one missing loop in HV5 as compared to the isolate with Acc. No. NC_003623 (Fig. 4A) and even more structural changes in HV5 as compared to two other isolates.

The 3'-NCR has been shown to affect the symptom expression in tobamovirus infections (Rabindran *et al*., 2005). However, no such evidence is available for nepoviruses. Viruses from the family *Comoviridae* share many structural and functional features with those from the family *Potyviridae*. In case of potyviruses, the 3'-terminal part of genome (the last 200 nts of CP gene and 3'-NCR) contains several sequences and secondary structures required for proper virus genome replication (Haldeman-Cahill *et al*., 1998). Mutational analysis of the 3'-NCR region of Tobacco vein mottling virus (potyvirus) outlined its role in symptom expression (Rodríguez-Cerezo *et al*., 1991).

Therefore, it is tempting to assume that nucleotide differences in the RNA2 3'-NCR of the HV5 isolate as compared to other isolates, leading to substantial conformational changes of the secondary RNA structure, may be related to particular biological behavior (mild or asymptomatic reactions) of this isolate. However, to determine definitely the genome part(s) responsible for symptom attenuation and to confirm our assumption, it will be necessary to construct an infectious cDNA clone for GFLV and carry out a mutational analysis.

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