SHORT COMMUNICATION

GENETIC VARIABILITY OF *GRAPEVINE FANLEAF VIRUS* ISOLATES WITHIN GENES $1B^{HEL}$ AND $1E^{POL}$

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SUMMARY

The genetic variability of six isolates of *Grapevine fanleaf virus* (GFLV) from South Moravia (Czech Republic) and Italy was investigated by RT-PCR and sequencing within part of the RNA-1-encoded genes $1B^{Hel}$ (379 nts) and $1E^{Pol}$ (365 nts). Sequence analyses showed 96-100% and 91.4-100% identity at the amino acid level for genes $1B^{Hel}$ and $1E^{Pol}$, respectively, among Czech, Italian and other isolates for which sequence information is available. As expected, some of the conserved motifs of the helicase and polymerase proteins were found in the deduced amino acid sequences, although isolates PN33 from Czech Republic, and isolates 55TK and UR11 from Italy had a Ile to Val subtitution in the helicase motif B. Phylogenetic analyses confirmed the close relationship among GFLV isolates and the relatedness of GFLV isolate WAPN173 with *Arabis mosaic virus* (ArMV) in protein $1B^{Hel}$. Two distinct phylogenetic clusters were identified for protein $1E^{Pol}$, with isolates from California and the French reference strain F13 grouping together, and isolates from the Czech Republic, Italy, New Zealand, and USA grouping in a different lineage. This study provides new insights into the genetic variability of GFLV RNA-1-encoded genes $1B^{Hel}$ and $1E^{Pol}$.

Key words: *Grapevine fanleaf virus*, molecular variability, helicase, polymerase, phylogeny.

Grapevine fanleaf virus (GFLV), genus *Nepovirus*, family *Secoviridae* is a severe disease agent of grapevines in many regions of the world. Crop losses up to 80% have been reported (Bovey, 1973). GFLV has a segmented genome consisting of two single-stranded, positive-sense RNA molecules that are encapsidated separately. Both genomic RNAs (RNA-1 and RNA-2) are covalently linked to a small viral protein (VPg) at their 5' end, are polyadenylated at their 3' terminus, and encode a single large polypeptide that is processed into functional proteins required to complete the virus life cycle. GFLV exists as various molecular variants in Europe, Africa, Middle East and North America (Naranghi-Arani *et al*., 2001; Vigne *et al*., 2004; Fattouch *et al*., 2005; Bashir *et al*., 2007; Pompe-Novak *et al*., 2007; Liebenberg *et al*., 2009; Mekuria *et al*., 2009; Oliver *et al*., 2010). Molecular variability has also been detected in viral isolates from the Czech Republic (Komínek *et al*., 2006; Eichmeier *et al*., 2010). Most of these studies have focused on RNA-2, whereas information on the genetic variability of RNA-1 is limited (Mekuria *et al*., 2009; Oliver *et al*., 2010; Wei and Clover, 2008), as shown also by the paucity of RNA-1 sequences deposited in GenBank.

The aim of this study was to advance our knowledge on the genetic variability of GFLV RNA-1 by characterizing the partial nucleotide sequence of genes $1B^{Hel}$ and $1E^{Pol}$ of six viral isolates from the Czech Republic and Italy.

The isolates used in this study were collected in 2008-2010. The four Czech isolates were recovered from grapevine cultivars grown in South Moravia for more than 20 years although they originated from Moldova (Table 1). The two Italian isolates were supplied by Dr. M. Digiaro (IAM Bari, Italy).

RT-PCR and sequencing was done as described (Eichmeier *et al*., 2010) with specific primers (Table 2). 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, using the BigDye Terminator v3.1 kit (Applied Biosystems, USA). Separation of the fragments was done on an ABI-PRISM 310 genetic analyser (Applied Biosystems, USA). Sequencing was done in both directions using primers in Table 2.

DNA amplicons of part of gene $1B^{Hel}$ of the six GFLV isolates, were obtained by RT-PCR of a RNA-1 segment between nucleotide (nt) positions 2653 and 3032, with respect to the reference strain F13 (GenBank accession No. NC_003623). Nucleotide sequences were analysed with CLC Main Workbench 5.0 (CLC bio,

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Table 1. RNA-1 sequences of *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV) used in this study.

Virus	RNA1	Accession for nucleotide	Accession for protein	Host	Cultivar	Isolate	Size (nt)	Country of origin	Reference
GFLV	full RNA1	NC_003615	NP_619689	*V. vinifera*	Muscat	F13	7342	France	Ritzenthaler *et al.*, 1991
GFLV	partial $1B^{Hel}$	HM130545		*V. vinifera*	Kodrjanka	KO1	379	Czech Republic	This study
GFLV	partial $1B^{Hel}$	HM130546		*V. vinifera*	Pamjati Negrula	PN32	379	Czech Republic	This study
GFLV	partial $1B^{Hel}$	HM130547		*V. vinifera*	Pamjati Negrula	PN33	379	Czech Republic	This study
GFLV	partial $1B^{Hel}$	HM130548		*V. vinifera*	Pamjati Negrula	PN35	378	Czech Republic	This study
GFLV	partial $1B^{Hel}$	HM130549		*V. vinifera*	Cinsaut Blanc	55TK	379	Italy (Turkisch origin)	This study
GFLV	partial $1B^{Hel}$	HM130550		*V. vinifera*	URS	UR11	378	Italy (unknown origin)	This study
GFLV	partial $1E^{Pol}$	HM130551		*V. vinifera*	Kodrjanka	KO1	364	Czech Republic	This study
GFLV	partial $1E^{Pol}$	HM130552		*V. vinifera*	Pamjati Negrula	PN32	367	Czech Republic	This study
GFLV	partial $1E^{Pol}$	HM130553		*V. vinifera*	Pamjati Negrula	PN35	365	Czech Republic	This study
GFLV	partial $1E^{Pol}$	HM130554		*V. vinifera*	Kišmiš Lucistyj	KML51	364	Czech Republic	This study
GFLV	partial $1E^{Pol}$	HM130556		*V. vinifera*	URS	UR11	364	Italy (unknown origin)	This study
GFLV	partial $1E^{Pol}$	HM130555		*V. vinifera*	Cinsaut Blanc	55TK	365	Italy (Turkisch origin)	This study
GFLV	partial $1E^{Pol}$	EU741689	ACF77020	*Chenopodium quinoa*	*quinoa* Willd.		345	New Zealand	Wei and Clover, 2008
GFLV	partial $1E^{Pol}$	EU741690	ACF77021	*V. vinifera*			345	USA	Wei and Clover, 2008
GFLV	full $1E^{Pol}$	GU972569	ADJ10926	*V. vinifera*	Cabernet Savignon	CACSC3	2511	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972568	ADJ10925	*V. vinifera*	Cabernet Sauvignon	CACSC2	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972566	ADJ10923	*V. vinifera*	Cabernet Sauvignon	CACSC2	2061	USA	Oliver *et al.*, 2010
GFLV	full $1E^{Pol}$	GU972562	ADJ10919	*V. vinifera*	Zinfandel	CAZINA5	2511	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972559	ADJ10916	*V. vinifera*	Zinfandel	CAZINA2	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972567	ADJ10924	*V. vinifera*	Cabernet Sauvignon	CACSC1	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972560	ADJ10917	*V. vinifera*	Zinfandel	CAZINA3	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972561	ADJ10918	*V. vinifera*	Zinfandel	CAZINA4	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972558	ADJ10915	*V. vinifera*	Zinfandel	CAZINA1	2061	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972565	ADJ10922	*V. vinifera*	Cabernet Sauvignon	CACSB3	2008	USA	Oliver *et al.*, 2010
GFLV	partial $1E^{Pol}$	GU972585	ADJ10942	*V. vinifera*	Cabernet Sauvignon	CACSB1a	1001	USA	Oliver *et al.*, 2010
GFLV	full RNA1	GQ332372	ACZ58632	*V. vinifera*	Pinot Noir	WAPN173	7342	USA	Mekuria *et al.*, 2009
GFLV	full RNA1	GQ332373	ACZ58633	*V. vinifera*	Pinot Noir	WAPN6132	7342	USA	Mekuria *et al.*, 2009
ArMV	full RNA1	AY303786	AAQ73821	*V. vinifera*	Pinot Gris	NW	7334	Germany	Wetzel *et al.*, 2004

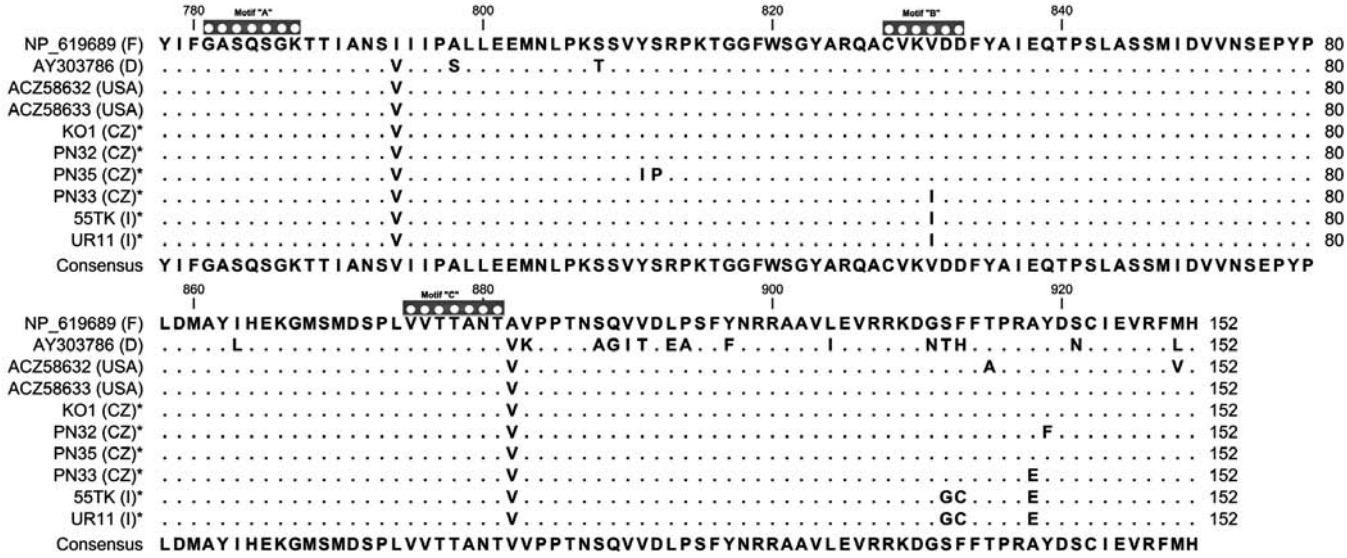

Fig. 1. Multiple alignment of deduced GFLV 1B^{Hel} amino acid sequences with conserved motifs A, B and C. The sequences obtained in this study are marked by asterisks.

Denmark), and deposited in GenBank as accession Nos HM130545 - HM130550.

Sequence alignments of the partial gene 1B^{Hel} of Czech, Italian and other virus isolates for which sequence information is available (Table 1), showed a 86.4-98.9% and 96-100% identity at the nucleotide and amino acid (aa) levels, respectively. The amplified region is part of gene 1B^{Hel}, that constitutes a P-loop containing nucleoside triphosphate hydrolase (Marchler-Bauer *et al*., 2008). As expected, the conserved Walker A (GxxxxGK[S/T]) and Walker B (hhhh[D/E]) motifs, with "h" being a hydrophobic residue, were found in the deduced amino acid sequences of all GFLV isolates (Fig. 1). Similarly, the helicase motifs A, B and C described by Gorbalenya *et al*. (1990) were conserved among the isolates, although isolates PN33 from the Czech Republic, and isolates 55TK and UR11 from Italy had a I (Ile) to V (Val) substitution in motif B (Fig. 1). The phylogenetic analysis using the neighbor-joining method of the CLC Main Workbench 5.0 package (CLC bio, Denmark) confirmed the close relationship among GFLV isolates and the relatedness of isolate WAPN173 with *Arabis mosaic virus* (ArMV) (Fig. 2).

DNA amplicons of the partial gene 1E^{Pol} of the six GFLV isolates were obtained by RT-PCR of RNA-1 between nt positions 4623-5627, with respect to the reference strain F13 (GenBank accession No. NC_003623), were sequenced and deposited in GenBank as accession Nos HM130551 - HM130556. Sequence alignments showed a 79.5-100% and 91.4-100% identity at the nt and aa levels, respectively (Fig. 3). The Czech isolates had five residue mutations compared with strain F13, i.e. D to E at position 1605, N to S at position 1623, A to

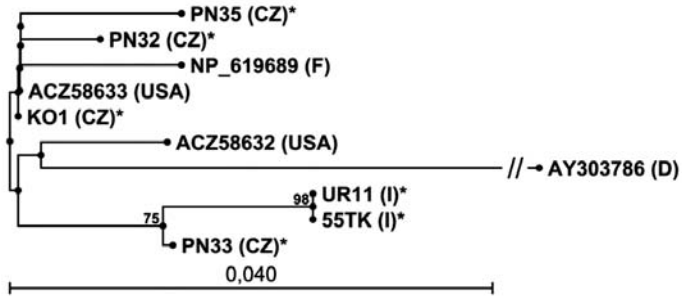

Fig. 2. Phylogenetic tree constructed with amino acid sequences of proteins encoded by GFLV gene 1B^{Hel} (neighbor-joining method with 100 bootstrap replicates).

Table 2. Primers used in this study.

Primer	Length (nt)	Sequence (from 5' to 3')	Direction	Localization in RNA-1 (nt)	Gene
CAEH2	24	TGGCAGGAGAGTTTGCAGACCTCT	forward	1328-1351	1B^{Hel}
EACH4	22	GAGAACTGCAACAGCCTCACTT	reverse	3114-3135	1B^{Hel}
CAER3	23	CCCAAAAGTCATCGCAATGCTTG	forward	4536-4558	1E^{Pol}
CAER4	21	CTACTCGATGAAGTTGCAGGT	forward	4896-4917	1E^{Pol}
EACR3	22	TTGATGCCCACTTGACAAGGCA	reverse	5351-5372	1E^{Pol}

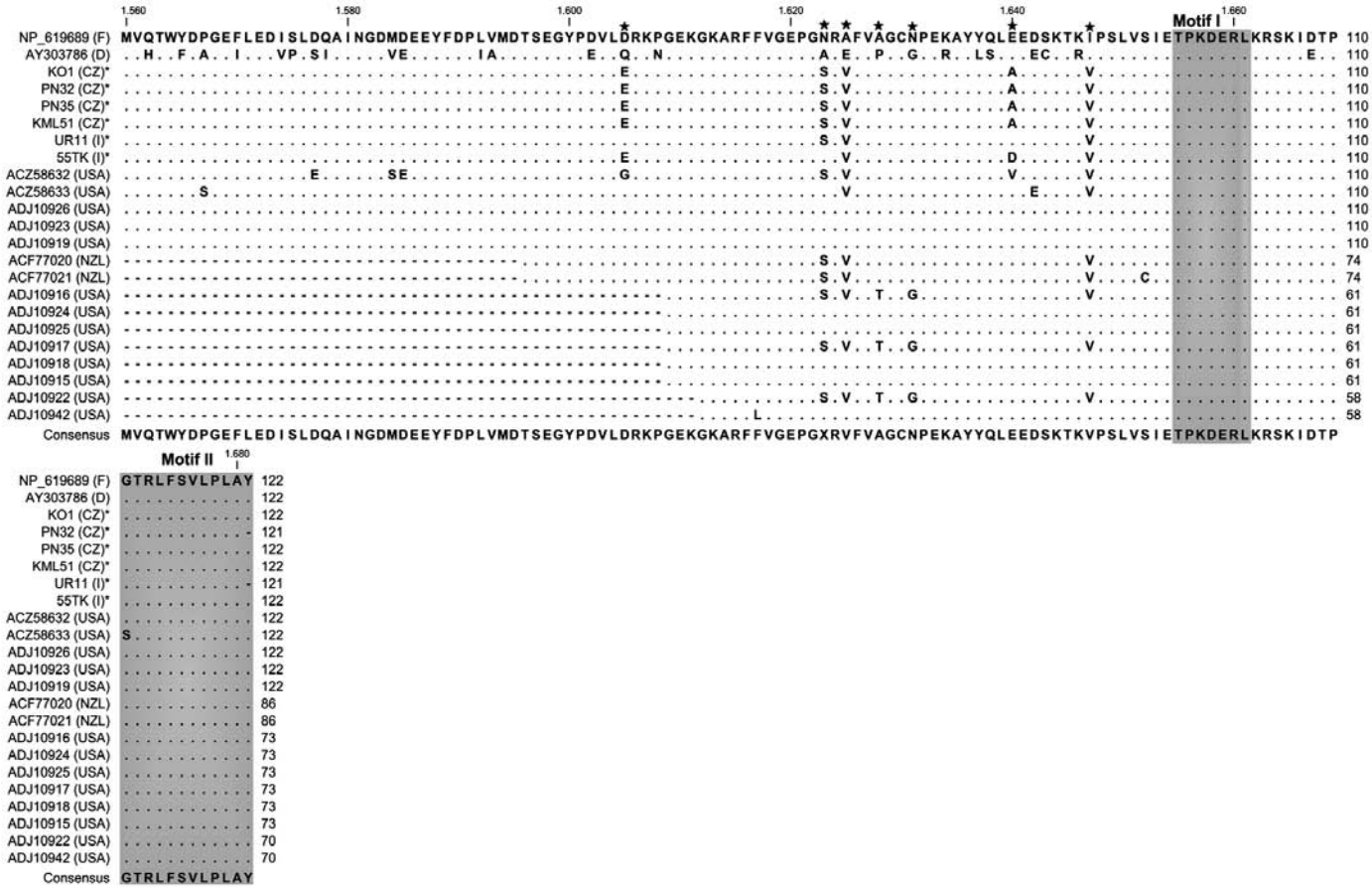

Fig. 3. Multiple alignment of deduced GFLV $1E^{Pol}$ amino acid sequences with conserved motifs I and II. Asterisks above amino acids indicate the most variable positions. The sequences obtained in this study are marked by asterisks.

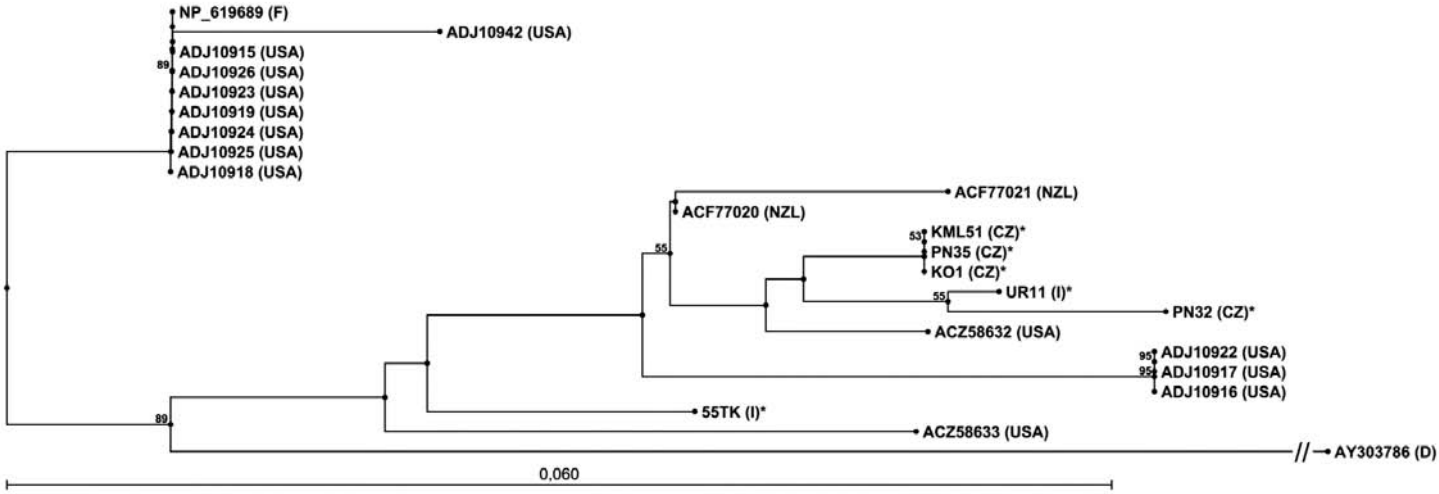

Fig. 4. Phylogenetic tree constructed with amino acid sequences of proteins encoded by GFLV gene $1E^{Pol}$ (neighbor-joining method with 100 bootstrap replicates).

V at position 1625, E to A at position 1640, and I to V at position 1647. As expected, two of the eight conserved polymerase motifs (Candresse *et al*., 1990; Koonin and Dolja, 1993) were found in the deduced aa sequences of the six GFLV isolates and others for which sequence information is available in GenBank, as well as for ArMV (Fig. 3). Isolate WAPN6132 from the USA had a G to S substitution in conserved motif II. In a phylogentic tree constructed with the aa sequence of the partial $1E^{Pol}$ gene viral isolates from California clustered with the French reference strain F13, whereas isolates from the Czech Republic, Italy, New Zealand, and other isolates from the USA grouped in a different clade (Fig. 4).

Our results show that GFLV genes $1B^{Hel}$ and $1E^{Pol}$ are relatively conserved among isolates from various geographic origins, including the Czech Republic and Italy, and from different grapevine cultivars. A slightly wider divergence due to individual amino acid substitutions was observed at the aa level in gene $1E^{Pol}$ (94.1-100%) compared with gene $1B^{Hel}$ (96-100%). This study expands on earlier research on the genetic variability of GFLV RNA-1 and provides new insights into genes $1B^{Hel}$ and $1E^{Pol}$ for which limited information was available.

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