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SHORT COMMUNICATION

Genetic variability within RNA2 of *Grapevine fanleaf virus*

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Abstract The genetic variability of *Grapevine fanleaf virus* (GFLV) was assessed within RNA2 of nine isolates from *Vitis vinifera* cv. Volovnik in a vineyard in Slovenia by immunocapture (IC)–reverse transcription (RT)–polymerase chain reaction (PCR)–restriction length fragment polymorphism (RFLP), followed by cloning and sequencing. Four, one, and nine distinct *Sty*I restrictotypes were identified in the $2A^{HP}$, $2B^{MP}$, and $2C^{CP}$ genes, respectively, by IC–RT–PCR–RFLP. Each isolate had a specific *Sty*I RFLP profile across the three RNA2-encoded genes. Sequence analysis of cloned RNA2 ORF amplicons obtained by IC–RT–PCR showed mixed infection in four of the nine isolates and a slightly higher nucleotide variability in the $2A^{HP}$ and $2C^{CP}$ genes relative to the $2B^{MP}$ gene. Also, gene $2A^{HP}$, unlike genes $2B^{MP}$ and $2C^{CP}$, had a variable size (765–774 nucleotides) and high amino acid diversity (up to 15%). In addition, a recombination event was identified at nucleotide position 220–225 of gene $2A^{HP}$ in three of the nine isolates. No clear association was apparent between symptomatology and restrictotype composition, phylogenetic clustering, or occurrence of recombination. This study provides new insights into the genetic diversity of GFLV.

Keywords GFLV · Nepovirus · Recombination · RFLP · RNA2 variability

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Grapevine fanleaf virus (GFLV) is a nepovirus responsible for fanleaf degeneration disease in grapevines, causing yield losses of up to 80%, poor fruit quality and reduced grapevine longevity (Andret-Link, Schmitt-Keichinger, Demangeat, Komar, & Fuchs, 2004; Martelli & Savino, 1990; Raski, Goheen, Lider, & Meredith, 1983). It

is naturally transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* and also by vegetative propagation and/or grafting (Andret-Link et al., 2004; Martelli & Savino, 1990; Raski et al., 1983).

The genome of GFLV is composed of two single-stranded positive sense RNA molecules (RNA1 and RNA2) (Fig. 1). Each of the two genomic RNA encodes a polyprotein, which is processed into functional proteins by the RNA1-encoded protease (Andret-Link et al., 2004). RNA2 includes a single open reading frame (ORF) of 3,324–3,333 nucleotides that is composed of three genes ($2A^{HP}$, $2B^{MP}$ and $2C^{CP}$) (Serghini et al., 1990; Vigne, Demangeat, Komar, & Fuchs, 2005; Wetzel, Meunier, Jaeger, Reustle, & Krczal, 2001), which code for a homing protein (HP), movement protein (MP) and coat protein (CP), respectively (Fig. 1).

The genetic diversity of GFLV has been assessed recently in France (Vigne, Bergdoll Guyader, & Fuchs, 2004b), Tunisia (Fattouch et al., 2005), and the USA (Naraghi-Arani, Daubert, & Rowhani, 2001). These studies focused on the characterization of the complete (Naraghi-Arani et al., 2001; Vigne et al., 2004b) or partial (Fattouch et al., 2005) $2C^{CP}$ gene by immunocapture (IC)–reverse transcription (RT)–polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) combined with sequencing after cloning. The co-existence of different GFLV variants was described in some isolates with a nucleotide (nt) sequence diversity ranging from 0.5 to 13.8%, indicating a quasispecies structure (Naraghi-Arani et al, 2001). In addition, the ORF (Vigne et al., 2005) and complete RNA2 sequences (Serghini et al., 1990; Wetzel et al., 2001) of a few GFLV isolates were determined. Furthermore, recombination events were detected in the $2B^{MP}$ and $2C^{CP}$ genes (Vigne et al., 2004b, 2005).

In this work, we expanded on previous genetic diversity studies by characterizing the RNA2 ORF of nine GFLV isolates from *Vitis vinifera* cv. Volovnik in a vineyard (Lože) in Slovenia. The aim of our study was to explore variability beyond the $2C^{CP}$ gene by targeting the entire RNA2 ORF, including the $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes. Our objective was to examine the diversity of each gene at the nucleotide and amino acid (aa) levels, and determine its contribution to genetic variability, as well as characterize the occurrence of new recombination events. We also attempted to correlate disease symptoms and genetic diversity.

Infected grapevines were selected based on typical fanleaf disease symptoms and the presence of GFLV, as shown by DAS-ELISA. Test plants consisted on *Vitis vinifera* cv. Volovnik grafted onto rootstock SO4 (*V. berlandieri* × *V. riparia*). The presence of *Xiphinema index* was tested twice in vineyard soil samples (Urek, Širca, & Karssen., 2005) but was not detected. The nine GFLV isolates that were selected for this study were further characterized by IC–RT–PCR–RFLP and sequencing.

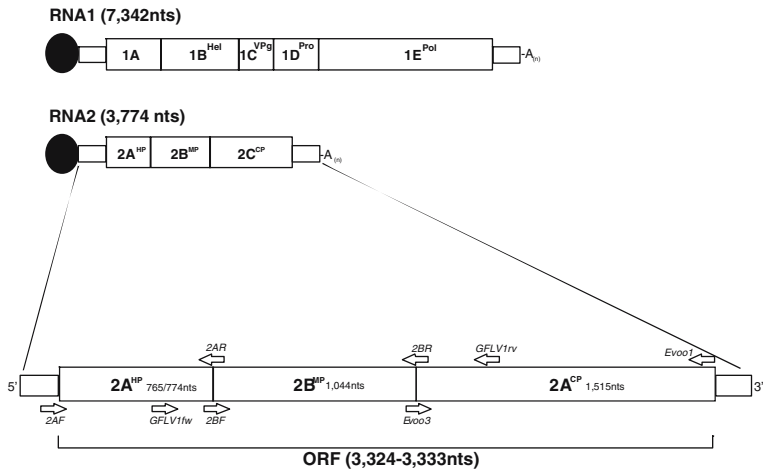

Fig. 1 Genetic organization of GFLV RNA1 and RNA2. ORFs are represented by wide open boxes, the small covalently linked viral protein (VPg) by a black circle, and the 5′ and 3′ non-coding regions by narrow boxes. The size of the RNA2 ORF, $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes is indicated, as well as the relative position of the oligonucleotides used in this study

DNA products obtained by IC–RT–PCR using primer Evoo1 for the RT step and specific primer pairs for the PCR amplification of the GFLV RNA2-encoded $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes (Table 1, Fig. 1) were analyzed by RFLP with *Sty*I (Fig. 2). A total of 14 restrictotypes were detected among all isolates. The $2C^{CP}$ gene featured the highest variability with nine different restrictotypes (T, Z, V, O, J, S, M, P, R) followed by gene $2A^{HP}$ with four restrictotypes (I, B, H, N), and gene $2B^{MP}$ with only one restrictotype (A) (Table 2). Interestingly, the restrictotype composition across the three genes was specific to each GFLV isolate (Table 2). Also, mixed infection by several restrictotypes in at least one of the RNA2-encoded genes was found for all the GFLV isolates, except Vol52 (Table 2).

In order to further analyze genetic diversity, the complete RNA2 ORF of the nine GFLV isolates was amplified by IC–RT–PCR, cloned into pGEMT vector and sequenced. The number of clones sequenced per isolate ranged from 1 to 6, reaching a total of 28. In silico RFLP analysis indicated the presence, among the sequenced clones, of restrictotypes B, I and H for gene $2A^{HP}$; restrictotype A for gene $2B^{MP}$; and restrictotypes V, T, S, R, Z and P for gene $2C^{CP}$. It also confirmed the presence of at least two distinct

Table 1 Oligonucleotides used in this study

Primer	Sense	Sequence 5′–3′	Position (nts)
2AF	Forward	CTYTTAYTTTGCGCTTTATTTGYTTAG	145–171
2AR	Reverse	CAATHGTYTGCCCMCCRGTA	1018–1037
2BF	Forward	CTACYGGKGGGCARACDATT	1017–1037
2BR	Reverse	GGTTGARCTCARYCTRGGCT	2022–2041
Evoo3	Forward	ACTGTCTAGAGGATTRGCYGGYAGAGGAGT	2045–2067
Evoo1	Reverse	GACTATCTAGACACATATATACACTTGGGTCTTTTAA	3574–3600
GFLV1fw	Forward	CCACTTGGAGGARGTGGTATGAC	771–793
GFLV1rv	Reverse	GRTGGATRAGCCARTGBGGRAC	2321–2342

The position refers to the RNA2 sequence of GFLV isolate F13 (Serghini et al., 1990)

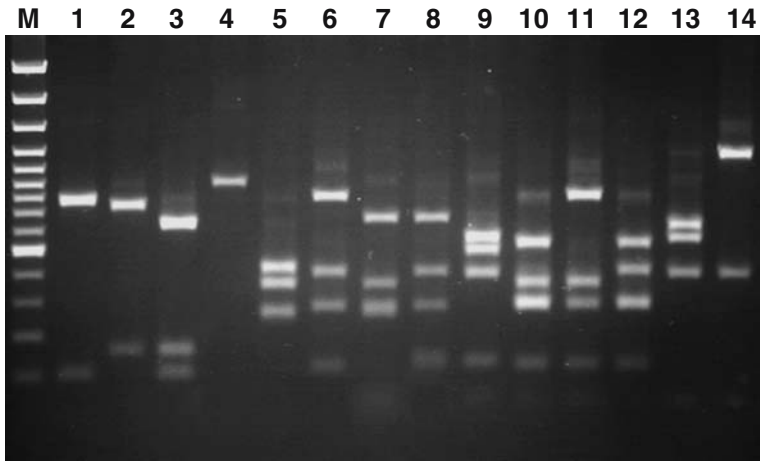

Fig. 2 IC–RT–PCR–RFLP electrophoretic patterns of Slovene GFLV isolates. Lanes 1–4, isolate Vol57 with restrictotype B, isolate Vol54 with restrictotype H, isolate Vol47 with restrictotype I, and isolate Vol55 with restrictotype N in the $2A^{HP}$ gene, respectively; lane 5, isolate Vol57 with restrictotype A in the $2B^{MP}$ gene; lanes 6–14, isolate Vol55 with restrictotypes J, isolate Vol55 with restrictotype M, isolate Vol47 with restrictotype O, isolate Vol57 with restrictotype P, isolate Vol57 with restrictotype R, isolate Vol54 with restrictotype S, isolate Vol55 with restrictotype T, isolate Vol47 with restrictotype V, and isolate Vol55 with restrictotype Z in the $2C^{CP}$ gene, respectively; and lane M, a DNA ladder (Fermentas) as size standard

Table 2 Association between symptoms of nine GFLV isolates from *Vitis vinifera* cv. Volovnik in vineyard Lože in Slovenia and restrictotype distribution, phylogenetic relationships, and recombination in gene $2A^{HP}$

Restrictotype				Phylogenetic group	Recombination in gene $2A^{HP}$	GFLV symptoms[a]						
Isolate	$2A^{HP}$	$2B^{MP}$	$2C^{CP}$			N	L	S	I	F	Z	B
Vol45	B,H,I	A	T,Z	2	–	+	+	+	–	+	+	–
Vol47	B,I,N	A	T,V,O	3	+	+	+	+	+	–	–	+
Vol49	H,I	A	T,V,J	3	+	+	+	+	–	+	–	–
Vol50	H,I	A	V	3	+	+	+	+	+	–	–	–
Vol51	B,H	A	T,V	1,2	–	+	+	–	+	+	+	–
Vol52	H	A	T	1	–	+	+	+	–	–	–	+
Vol54	B,H,I	A	V,S	1,2	–	+	–	+	–	–	–	+
Vol55	B,H,N	A	T,V,J,M	1,2	–	+	+	+	–	–	+	+
Vol57[b]	B	A	V,P,R	1,2	–	nt	nt	nt	nt	nt	nt	nt

[a] N, Short internodes, double internodes, bifurcation on nodes and flat stem; L, asymmetrical leaves; S, shoot proliferation; I, bifurcation on internodes, F, fasciation of shoots, Z, zigzag growth; and B, shot or no berries. nt: not tested

[b] No symptoms were recorded because plant 57 died soon after the start of this study

restrictotypes in genes $2A^{HP}$ and $2C^{CP}$ of isolates Vol51, Vol54, Vol55 and Vol57.

The GFLV RNA2 ORF sequences were aligned and neighbour-joining trees built to explore phylogenetic relationships by using the Vector NTI-v7 package (InforMax, Inc.) and Clustal X v1.81 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). Three groups designated 1, 2 and 3 were obtained (Fig. 3A). Variants belonging to groups 1 and 2 had an average sequence similarity of 88.3 ± 0.1 and 93.9 ± 0.2% at the nt and aa levels, respectively. Mixed infection with variants from groups 1 and 2 was observed for isolates Vol51, Vol54, Vol55 and Vol57, thus confirming predictions from RFLP data. Variants in group 3 were more closely related to variants in group 2 than to those in group 1 (97.9 ± 0.1 vs 89 ± 0.2% nt sequence similarity), suggesting a potential recombination event within the RNA2 ORF of variants from group 3.

To further investigate the occurrence of recombination, the sequence of the $2A^{HP}$, $2B^{MP}$ and $2C^{CP}$ genes were separately aligned and neighbour-joining trees were built for each RNA2-encoded gene (data not shown). The average sequence diversity between variants from groups 1 and 2 in gene $2C^{CP}$ (13.2%) correlated well with the values (13 to 13.8%) previously reported (Naraghi-Arani et al., 2001; Vigne et al. 2004b). The tree corresponding to the alignment of the $2A^{HP}$ gene sequences showed the same three groups as for the alignment of the RNA2 ORF sequences. However, variants of group 3 were more closely related to those in group 1 (92.4 ± 0.3% nt sequence similarity) and differed substantially more from those in group 2 (95.1 ± 0.3% nt sequence similarity) than in the case of the RNA2 ORF alignments. Phylogenetic trees derived from the alignments of the $2B^{MP}$ and $2C^{CP}$ genes showed variants of group 3 as part of group 2 (data not shown).

The suspected recombination event in gene $2A^{HP}$ of variants from group 3 was confirmed when both RNA2 ORF and $2A^{HP}$ gene sequences were analyzed with the recombination detection programme 2 (RDP2) package (Martín and Rybicki, 2000) (Fig. 3B). The different tools of the RDP2 package (RDP, Bootscan, Geneconv, Maxchi, Chimaera and SiScan) detected the recombination event and predicted a recombination junction close to position 225 of gene $2A^{HP}$ (Fig. 3B). Putative parental lineages of these recombinants were each from groups 1 and 2, thus confirming predictions from sequence similarities and phylogenetic relationships. Recombination events were described previously in the $2B^{MP}$ and $2C^{CP}$ genes (Vigne, Komar, & Fuchs, 2004a, 2004b; 2005) but not in the $2A^{HP}$ gene. Interestingly, grapevines infected with GFLV recombinants in the $2A^{HP}$ gene did not show different or more severe symptoms in comparison to grapevines infected with non-recombinant GFLV isolates (Table 2). Similar observations

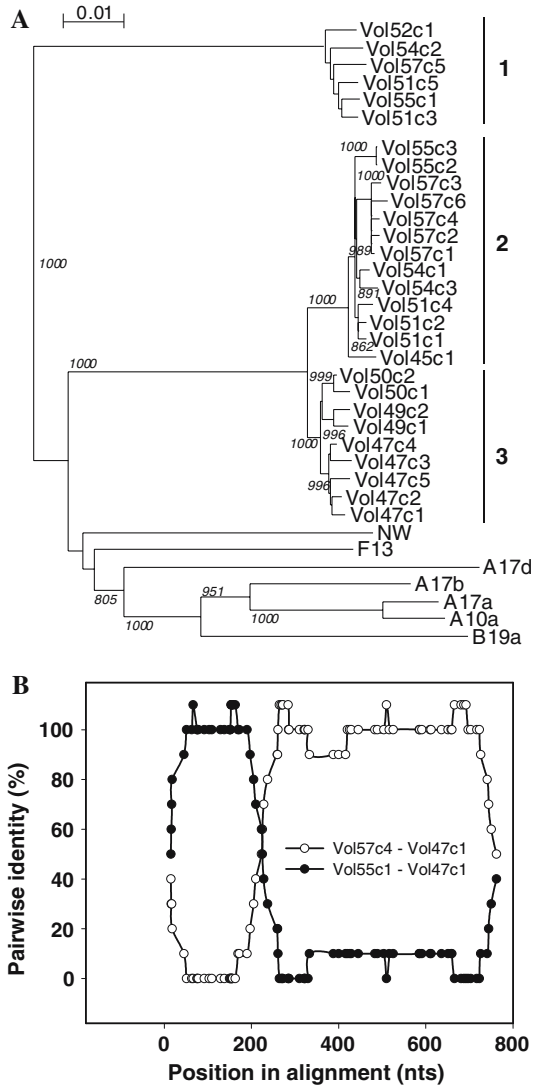

Fig. 3 (**A**) Neighbour-joining tree for the alignment of the GFLV RNA2 ORF sequences of isolates from Slovenia, isolates A10a (GenBank accession number AY780902), A17a (AY780899), A17b (AY780900), A17d (AY780901), B19a (AY780903) and F13 (NC003623) from France, and isolate NW (AY017338) from Germany. Bootstrap values higher than 800 are denoted in the trees. The three Slovene phylogenetic groups are labelled 1, 2, and 3. The sequences are denoted with the name of the isolate followed by the clone number, e.g. Vol47c1 is the first clone of isolate Vol47. The Slovene GFLV RNA2 ORF sequences were made available in GenBank as accession numbers DQ922652-DQ922679. (**B**) Recombination in GFLV RNA2-encoded $2A^{HP}$ gene detected by the RDP tool of the RDP2 package. The two sequences, which best fitted as parental lineages (Vol57c4 and Vol55c1), were aligned with the sequence of recombinant variant Vol47c1

were reported in herbaceous hosts for a GFLV recombinant isolate in the $2C^{CP}$ gene (Vigne et al., 2005). In addition, no clear association was apparent between GFLV symptoms in cv. Volovnik and variant composition of the infecting isolate (Table 2).

The RNA2 ORF sequence of the nine GFLV isolates from Slovenia and seven other isolates, for which information is available in GenBank (A10a, A17a, A17b, A17d, B19a, F13 and NW), were aligned and phylogenetic trees were built. None of the sequences from the seven isolates outside of Slovenia co-localized in any of the three groups defined in this study (Fig. 3A). Alignments of one representative ORF sequence from each of the three Slovene groups and the other RNA2 ORF sequences indicated similar sequence similarity values as those shared among Slovenian groups, ranging from 87 to 91% and 93 to 96% at the nt and aa levels, respectively. The $2A^{HP}$ and $2C^{CP}$ genes showed the highest nt sequence variability, reaching in many cases similarity values close to 87%, in comparison to the $2B^{MP}$ gene, for which similarity values were close to 90%.

The $2A^{HP}$ gene and its encoded protein varied in size from 765 bp (255 aa) for isolate A17d, 771 bp (257 aa) for isolate F13 and Slovenian variants from group 1, to 774 bp (258 aa) for isolates A10a, A17a, A17b, B19a, NW and Slovenian variants from groups 2 and 3. The region of protein $2A^{HP}$ responsible for the variability in size is located close to the N-terminus, within aa 49–108. Limited aa sequence identity was found in this region in comparison to the whole protein (51.7 vs 70.9%). Similar observations have been reported previously for the $2A^{HP}$ gene of *Arabic mosaic virus* (ArMV), another member of the genus *Nepovirus* (Wetzel, Fuchs, Bobko, & Krczal, 2002). The size of protein $2A^{HP}$ of 19 ArMV isolates ranged from 233 to 280 aa. In agreement with our findings, the N-terminus of ArMV protein $2A^{HP}$ was most variable while the C-terminus was more conserved. We also noticed that, in contrast to $2B^{MP}$ and $2C^{CP}$ genes, the $2A^{HP}$ gene of the studied isolates showed similar or lower sequence similarity values at the aa than

at the nt level, reaching values of as low as 84.9% aa sequence diversity. Limited information is available on the function(s) of protein $2A^{HP}$ in the GFLV replication cycle. Protein $2A^{HP}$ is necessary but not sufficient for replication of RNA2 and localizes in the replication site (Gaire, Schmitt, Stussi-Garaud, Pinck, & Ritzenthaler, 1999). The biological significance of the high aa sequence diversity and heterogeneity in size featured by this protein remains to be determined.

In summary, our study provided new insights into the genetic variability of GFLV RNA2. The $2A^{HP}$ gene located at the 5′ end of RNA2 had a variable size and high sequence diversity, as documented earlier for ArMV (Wetzel et al., 2002). Also, a recombination event was detected in the GFLV $2A^{HP}$ gene. To the best of our knowledge, this is the first report of recombination in the N-terminus protein encoded by a nepovirus RNA2. In addition, no association was found between symptomatology and genetic variability within the RNA2 ORF, including the occurrence of recombination. These results suggested that viral determinants for symptomatology likely map to the RNA2 noncoding regions and/or to RNA1. Further investigations are needed to validate this hypothesis.

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