Molecular Characterization of Phylogenetically Distinct Isolates of *Grapevine fanleaf virus* from Iran Based on 2A^{HP} Gene

Nemat Sokhandan-Bashir · Azam Hooshmand ·
Afsaneh Delpasand-Khabazi

Received: 8 May 2011 / Accepted: 3 February 2012 / Published online: 4 March 2012
© Indian Virological Society 2012

Abstract Movement and coat protein genes from *Grapevine fanleaf virus* (GFLV) isolates have been characterized previously from Iran. In this study, an optimized reverse transcription polymerase chain reaction protocol was established to amplify RNA2 genomic segment corresponding to the hypothetical protein (2A^{HP}). The sequence of 2A^{HP} was compared with that of previously reported GFLV strains/isolates from other countries which showed 82–86% sequence identities. The 2A^{HP} gene from Iran appeared to be standing distinct from other isolates of GFLV when genetic distance- or parsimony-based phylogenetic analyses were carried out. The present study for the first time reports characterization of Iranian isolate of GFLV based on 2A^{HP} gene.

Keywords Hypothetical protein · 2A^{HP} gene · RT-PCR · ELISA · GFLV · Nepovirus

Grapevine fanleaf virus (GFLV) is the major reason of grapevine degeneration. It can cause losses up to 80%, reduce fruit quality, and shorten the longevity of vines in the vineyard [19]. GFLV belongs to the genus *Nepovirus* in the family *Sercoviridae* [31] and is transmitted from one grapevine to another specifically by the dagger nematode, *Xiphinema index* [14]. It is also transmitted mechanically by plant sap. Transportation of vine stock, especially of susceptible cultivars is believed to play a major role in dissemination of GFLV [12]. The virus infects *Vitis* spp.

and species of other genera in nature and possesses a wide experimental host range [15, 17].

Symptoms incited by GFLV include open petiole sinus, shark-toothed leaf edges, mosaic, vein yellowing, stem fasciations, and zigzagged stem, leaf distortion and shortened internodes [25]. The symptoms may vary depending on different host species and GFLV isolates so that leaf symptoms ranging from fanleaf to yellow mosaic, vein banding, line pattern, chlorotic ring spots and mottle have been described in numerous grapevine varieties in several countries [19]. Similarly, isolates differing in type and severity of symptoms have been described in herbaceous hosts [16, 39].

GFLV is serologically related to *Arabidopsis mosaic virus* (ArMV). This and other relationships between the two viruses have resulted in the assumption that the two viruses have come from the same origin, or even GFLV has been origin for ArMV [13]. Discovery of a cross-protecting potential between these two viruses has supported this theory [16].

GFLV particles are isometric and 30 nm in diameter. The viral genome is composed of two single-stranded positive polarity linear RNA (+ssRNA) segments with a small virus-genome linked protein (VPg) and poly (A) tail at their 5' and 3' end, respectively. Both RNA segments contain only one open reading frame (ORF) and codes for a polyprotein, which are processed proteolytically at defined dipeptide cleavage sites by the RNA1- encoded proteinase into functional proteins [23]. RNA1 codes for the proteins involved in virus replication [26] and for the viral proteinase [18]. VPg, proteinase and RNA-dependent RNA polymerase (RDRP) are the RNA1- encoded proteins. RNA2 codes for a protein (2A^{HP}) which is required for RNA2 replication and could act as homing protein [10], the movement protein (2B^{MP}) which is the constituent protein

N. Sokhandan-Bashir (✉) · A. Hooshmand ·
A. Delpasand-Khabazi
Department of Plant Protection, University of Tabriz,
51664 Tabriz, Iran
e-mail: sokhandan@tabrizu.ac.ir

of tubules observed in plasmodesmata [27], and the coat protein (2C^{CP}) [32]. The complete RNA 1 (7,342 nt) for strain F13 [27], the RNA2 (3,774 nt) for strain F13 [32] and the German isolates, NW [40] have been sequenced.

Because the virus is transmitted through the vine root-stock material it is essential to use only virus-free material for propagation. To that end, various methods have been developed for detecting GFLV which include ELISA [7], reverse transcription polymerase chain reaction (RT-PCR) [28], RNA oligo probe capture RT-PCR [8] and IC-RT-PCR [41]. In Iran, grapes are produced at the rate of 2.1 million tons annually on about 270,000 ha [17]. The North West region of Iran shares a major part of the produce and therein grape industry has been established. Previously, the MP and CP have been isolated and sequenced from a reasonable number of GFLV isolates from Iran, but there is yet no molecular information as to the HP gene of such isolates. The purpose of the present study has been to set up an RT-PCR protocol for isolation of HP coding region from the virus isolates prior to subjecting to cloning and sequencing in order to compare this genomic region with counterpart region of previously recorded isolates or strains of the virus. This study could support the distinctness of the Iran isolates of GFLV as has been concluded in the previous reports [34–36]. Also, this study could provide another detection method of GFLV based on the HP coding region.

Vineyards in the northwest region of Iran encompassing Tabriz, Bonab, Maragheh, Shabestar, Malekan, Shiramin, Sardroud, Kheldjan, Azershahr, Lahroud, Ahar, Oskoo, Aras, Ajabshir, and Fakhrabad were inspected during spring and summer 2007 and 6–7 leaves were sampled from the vines suspected to be infected by GFLV according to the GFLV symptoms [19, 25]. Each sample was divided into three portions one of which was tested by ELISA, another portion was subjected to total RNA extraction for RT-PCR and the remainder was dried on calcium chloride (CaCl₂) granules for preservation at –20°C.

The collected samples were tested by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) [3] and the indirect direct antigen coated- ELISA (DAC-ELISA) [5] by the use of polyclonal commercially available anti-GFLV antibody (Bioreba, Switzerland). A sample was regarded as positive if its average absorbance value at 405 nm wave length was at least twice that of the wells treated with healthy samples.

Total RNA was extracted from ELISA-positive samples by the use of “protocol 4” of [29] with scaling down to 0.1 g leaf sample. Finally, each RNA sample was dissolved in 50 µl sterile distilled water. RT-PCR was performed on ELISA-positive samples with typical GFLV symptoms. Reverse transcription was done by the use of primers Oligo d(T)₁₆, G0, or CP912. In PCR reactions, two combinations

of primers, 5'NC/G0 or 5'NC/GMPR1, were used. 5'NC (5'CCAAGAGTTT(A/G)(A/G)GAAACTCA3') and G0 (5'CAGAATTCCCTTACCCTCTC3') corresponding to nucleotides (nts) 109–128 and 881–900 of GFLV-F13 [32] were designed by [40] as the forward and reverse primers, respectively. Amplification by these primers was expected to produce a 792 bp fragment encompassing 126 nts of 5'UTR and 668 nts of 5' proximity of 2A^{HP} gene located on the virus RNA2 segment. The degenerate reverse primer GMPR1 (5'CCBMGSTTGAGYTCGACYRTGAGA3') corresponding to nucleotides 2,024–2,047 of GFLV-F13 RNA2 was the same primer used in our previous work for isolation of the MP gene [33]. The use of 5'NC and GMPR1 was expected to produce a fragment of 1,995 bp encompassing 181 nts of 5'UTR plus HP and MP genes. The primer cp912 [17] corresponds to nucleotides of 2,936–2,959 of GFLV-F13 RNA2.

RT-PCR reagents were purchased from Fermentas (Lithuania). The first strand cDNA synthesis was performed by the use of 0.25 µg Oligo d(T)₁₆, 10 pmol G0, or 10 pmol CP912 per 10 µl reaction mix that contained 1× reverse transcription buffer, 1 mM dNTPs, 10 Units RNasin, 100 Units M-MuLV reverse transcriptase, and 2.5 µl total RNA extract. First, a 5 µl solution containing primer and RNA sample was incubated at 70°C for 5 min followed by chilling on wet ice. Then, the remaining reagents were prepared in 5 µl volume and added to the chilled tube. The tube was left at room temperature for 15–20 min. First strand synthesis was then performed at 42°C for 60 min followed by inactivation of the reverse transcriptase at 70°C for 10 min before PCR reactions were prepared in 10 µl volume containing 0.5 Unit *Taq* DNA polymerase, 0.6 pmol of each primer and 2 mM MgCl₂. When the virus specific primer (G0 or CP912) was used at the first strand synthesis stage this primer was not added further in the PCR reaction. The 10 µl PCR reaction mix was combined with 2.5 µl of cDNA mix.

In PCR with 5'NC/G0 an initial denaturation step of 94°C for 1 min was followed by 35 cycles of 94°C for 30 s, 47°C for 30 and 72°C for 1 min. Finally, the polymerization was completed at 72°C for 7 min. In optimization of the amplification the annealing temperature was raised to 61°C with 2°C increment. PCR with 5'NC/GMPR1 was performed with a similar thermo profile except that the annealing was done at 58°C for 2 min and polymerization at 72°C for 2 min. PCR products were run on 1.2% agarose in 0.5× TBE, stained with 0.5 µg/ml ethidium bromide and documented as described in [30].

About 20 ng of each fragment amplified with the primer set 5'NC/G0 was ligated into pTZ57R/T (Fermentas, Lithuania) in 10 µl ligation volume containing 50 ng of the plasmid and 3 Units of T4 DNA ligase. Then, the reaction was incubated at 4°C overnight and 1 µl was used to

transform *Escherichia coli* DH5 α cells which were made competent according to [2]. Selection and screening of the transformed cells were performed by the use of ampicillin resistance, white-blue system and restriction analysis as described in our publications [35].

Recombinant plasmids containing the amplified fragment were subjected to sequencing in Macrogen (Seoul, Korea) by the use of M13F and M13R primers. Then, the sequencing data obtained from each reaction was first searched for the 5'NC and G0 primer sites, then the sequence containing the G0 primer was reverse complemented and aligned with the sequence resulting from the use of the forward primer to determine the complete sequence data for whole stretch of the amplified region. Thus, sequences of two clone isolates including 6D8 and D12 from samples Kh6 were successfully determined, deposited into GenBank and assigned the accession codes JN834010 and JN834011, respectively. Then, the sequences were aligned with the corresponding region of previously reported GFLV strains/isolates and ArMV-PV215 [42] (Table 1) by the use of Genedoc [20]. The exact matches (identities) between the isolates were also determined by GeneDoc.

Both distance-based and parsimony trees were generated by the use of Phylip package 3.65 [9]. In both cases, the aligned sequences (Phylip format) were submitted to Seqboot to incorporate bootstrap values in the analyses. For the distance-based tree, first distance matrix was generated by the Dnadist program wherein the Jukes-Cantor correction method was applied and multiple data analysis option was incorporated. The resulting file was subjected to Neighbour and the generated trees were subjected to Consense program to choose a consensus tree which was viewed by Treeview [22]. For parsimony tree, the bootstrapped file was first subjected to DNAPars followed by choosing a

consensus parsimonious from among the generated parsimony trees before viewing by treeview.

DAS- and/or DAC-ELISA on 300 grapevine samples were resulted in positive reactions with 88 samples which were collected from East and West Azarbaijan and Ardabil provinces. The number of tested samples and infected samples from each location are listed in Table 2. In some cases, where no positive reactions were obtained in DAS-ELISA subjected to DAC-ELISA of the samples resulted in positive signals. However, no GFLV was detected from Shabesatar, Azershahr, Ahar, Oskoo, Aras, and Ajabshir. Samples from these locations were in addition to the 300 samples which were collected from the infected locations.

Reverse transcription with G0 and subsequent PCR with 5'NC and G0 gave the expected approximately 800 bp fragment from only one sample, Kh6. However, cDNA synthesis by CP912 and subsequent PCR with 5'NC and G0 resulted in amplification of the 800 bp fragment from several samples including Kh11 (Tabriz), Kj12 and Kj18 (Kheldjan), and B15 (Bonab). When first strand cDNA was synthesized with CP912 and subsequent PCR was performed with 5'NC/GMPR1, the expected 1,995 bp fragment was amplified from several samples including T1 (Tabriz), Kj12, Kj14, Kj16, Kj17, and Kj18 (Kheldjan); B4, B12, and B15 (Bonab); and Ma13 (Marageh) (Fig. 1).

It appeared that virus specific primers (G0 or CP912) were more efficient in priming the first strand cDNA synthesis than Oligo d(T)₁₆ because when subsequent PCR was done by 5'NC/G0 primer combination, the amplification was only resulted from cDNA samples, which were synthesized by G0 or CP912. This could be because Oligo d(T)₁₆ supposedly primes from the very 3' end of the RNA molecule far away from 2A^{HP} coding region but the specific primers sit closer (CP912) or right at 2A^{HP} region (G0). Accordingly, under a relatively inefficient condition,

Table 1 Accessions and origins of *Grapevine fanleaf virus* isolates/strains whose sequence data were used in this study

Origin	Accession(s)	Isolate/Strain*	References
France	AY780899–AY780903	A17a, A17b, A17d, B19a	Vigne et al. (2004) [37]
France	Nc_003623	F13	Serghini et al. (1990) [32]
Slovenia	DQ922652–DQ922679	Vol45c1, Vol47c1–Vol47c5, Vol49c1, Vol49c2, Vol50c1, Vol50c2, Vol51c1–Vol51c5, Vol52c1, Vol54c1–Vol54c3, Vol55c1–Vol55c3, Vol57c1–Vol57c6	Pompe-Novak et al. (2007) [24]
Germany	AY090011	ArMV-PV215	Wetzel et al. (2002b) [42]
Germany	AY017338	GFLV-NW	Wetzel et al. (2001) [40]
Germany	AY017339	ArMV-NW	
USA	GU972571–GU972584	CAZINA1, CAZINA2, CAZINA3, CAZINA4, CAZINA5, CACSB1, CACSB2, CACSB3, CACSB4, CACSB5, CACSC1, CACSC2, CACSC3, CACSC4	Oliver et al. (2010) [21]
Iran	JN834010	6D8	This paper
Iran	JN834011	D12	This paper

* Names of isolates are written in an order respective to their accession numbers

Table 2 Characteristics of samples with positive reactions in ELISA to anti-GFLV polyclonal antibody

Location ^a	Symptoms	No. tested samples	No. positive samples ^b
Urmia	VB, LD	35	2
Jolfa	VB	2	1
Tabriz	LD, STL	40	3
Kheldjan	VB, LD, SID	60	25
Bonab	LD, VB, STL, OLS	10	5
Malekan	LD, VB	15	5
Sardroud	YM	27	6
Marageh	YM	20	5
Khalatpushan	VB, Mo, LD	46	20
Lahroud	VB, LD	15	8
Fakhrabad	VB, Mo, LM, OLS	30	8

^a Jola, Tabriz, Kheldjan, Bonab, Malekan, Sardroud, Marageh, and Khalatpushan are in East Azarbaijan; Urmia in West Azarbaijan; Lahroud and Fakhrabad in Ardabil province

^b If average absorbance value at 405 nm for a sample was at least twice that of the healthy control

LD leaf distortion, STL shark tooth leaf, VB vein-banding, SID shortened internodes, OLS open leaf sinus, YM yellow mosaic, LY leaf yellowing, Mo mosaic, LM leaf mottling

Fig. 1 RT-PCR amplification stretches of *Grapevine fanleaf virus* 2A^{HP} coding region by the use of the primer sets 5'NC/G0 (a) or 5'NC/GMPR1 (b). Lane 1 in both gels are loaded with 100 bp DNA ladder plus. a, lanes 2–6: ~800 bp products amplified from samples Kh6, Kh11, Kj12, B15, and Kj 18, respectively, lane 7: RT-PCR on healthy control. b, lanes 2–10: 1995 bp fragment resulting from amplifications from samples B4, B12, Ma13, Kj14, B15, Kj16, Kj17, Kj18, and J, respectively, lane 11: RT-PCR on healthy control

these specific primers have more chances of polymerizing the 5'NC site. For a similar reason, amplification with 5'NC/GMPR1 was achieved only when CP912 was used in the first strand cDNA synthesis.

Optimization was done to improve efficiency of the PCR wherein annealing appeared to be the most important factor. Amplification by the primer set 5'NC/G0 was poor and non specific at annealing temperature of 47°C. However, PCR was optimized by 2°C increment, in the annealing which resulted in amplification of an intense fragment (Fig. 1). Increase of annealing temperature beyond 59°C resulted in weaker amplifications. Although lowering MgCl₂ concentration can also help to increase specificity of the reaction it simultaneously decreases the polymerase fidelity. Accordingly, raising the annealing temperature

may be more helpful than decreasing Mg⁺⁺ concentration [4, 6].

As in the case of some other plant viruses, in studies on GFLV it often happens that the virus is not detected in all the symptomatic samples [1, 34–36]. Our previous study have revealed that local isolates of the virus possess distinct genotypes so that primers which were designed according to sequences of exotic GFLV isolates were less efficient in priming the local isolates. For instance, with the MP gene, the previously designed primers did not amplify the expected fragment from as many samples as was achieved with the primers based on the sequences of local isolates [33]. It should also be born in mind that there are over 60 viral species reported to infect grapevine [11] and many of such viruses have common symptoms such as vein banding, fanleaf, and yellow mosaic.

Ligation of the PCR products into pTZ57R/T and subsequent transformation into *E. coli* resulted in generation of white colonies which contained the recombinant plasmids carrying the GFLV 2A^{HP} gene as revealed by restriction analysis of such plasmids. When subjected to sequencing only two clones (isolates) from sample Kh6 including 6D8 and D12 produced good sequencing data. Interestingly, it was revealed that the primer 5'NC can prime from two sites because the corresponding region on the virus RNA2 is repeated. In clone D12, the priming has started 25 nts downstream of the priming site for clone 6D8. Alignment of the sequences of 2A^{HP} clones reconfirmed that the amplified fragment corresponded to 2A^{HP} coding region of GFLV RNA2.

When compared to previously reported 2A^{HP} sequences from other parts of the world, overall 82–86% identities were found between the Iran isolates and the others.

Fig. 2 A parsimony tree based on 622 nucleotide sequence of 5' proximity of GFLV HP gene. The Iran HP isolates are marked. The bootstrap values are shown on the nodes. Branches with <50% bootstrap values are unresolved. The scale bar represents 10% bootstrap value and branch lengths are proportional to bootstrap values

The least similar isolate to the Iran isolates was CAZINA1 (USA) with 82% similarity whereas the closest isolate was Vo151C4 (Slovenia) with 86% identity. In most cases, the similarities between the Iran isolates and those from other countries ranged from 84 to 85% at the 2A^{HP} gene level.

Phylogenetic analyses revealed that the GFLV 2A^{HP} clone from Iran placed in a distinct position on the distance-based tree or parsimony tree (Fig. 2). Our previous analyses on the MP and CP genes have also demonstrated that Iran GFLV isolates are placed in a distinct position on the phylogenetic trees [33–36]. Also, our studies have

shown that the Iran isolates are the most divergent among the all the reported GFLV variants from around the globe. This might give other supports to the previous hypothesis that GFLV has originated in Iran and from there it has spread to other parts of the world [38] because such a distinct and divergent population of GFLV might be suggestive a long existence of the virus in this region.

We successfully established an RT-PCR protocol for isolation of 2A^{HP} gene from GFLV isolates from Iran for the first time. Subsequently, cloning and sequencing of such clones provided insights into molecular characteristics

of the virus at 2A^{HP} coding region. Success in the amplification of the 2A^{HP} coding region is an important achievement because this opens up new avenues to make new primers and hunt more GFLV isolates from the local vineyards. Molecular characterization of the virus genomic segments is very important as to development of molecular detection method and control of virus. Also, it is required in design and implementation of an effective protection strategy against the virus. Because 2A^{HP} is involved in the virus replication it might be a potential target in the virus control. Further, the distinctness of the Iran isolates based on the 2A^{HP} gene supported the conclusions we had drawn from our previous studies.

Acknowledgments We appreciated the research fund provided by research management office of The University of Tabriz. We also thank Dr. Thierry Wetzel for kindly providing the primers 5'NC and G0.

References

- Anofka GH, Shahrour W, Nakhla MK. Detection and molecular characterization of *Grapevine Fanleaf virus* and *Grapevine Leafroll-associated virus 3* in Jordan. *J Plant Pathol.* 2004;86: 203–7.
- Chung CT, Niemela SA, Miller RH. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci USA.* 1989; 86:2172–5.
- Clark MF, Adams AN. Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J Gen Virol.* 1977;34:475–83.
- Dieffenbach CW, Dveksler GS. PCR primer: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1995. p. 714.
- Dijkstra J, De Jager CP. Practical plant virology: protocols and exercises. The Netherlands: Springer; 1998. p. 459.
- Echols H, Goodman MF. Fidelity mechanisms in DNA replication. *Annu Rev Biochem.* 1991;60:477–511.
- Esmenjaud D, Walter B, Minot JC, Voisin R, Cornuet P. Biotin-avidin ELISA detection of grapevine fanleaf virus in the vector nematode *Xiphinema index*. *J Nematol.* 1993;25:401–5.
- Fattouch S, M'hirsi S, Acheche H, Marrakchi M, Marzouki N. RNA oligoprobe capture RT-PCR, a sensitive method for the detection of grapevine fanleaf virus in Tunisian grapevines. *Plant Mol Biol Reporter.* 2001;19:235–44.
- Felsenstein J. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Seattle: University of Washington, Department of Genome Sciences; 2004.
- Gaire F, Schmitt C, Stussi-Garaud C, Pinck L, Ritzenthaler C. Protein 2A of grapevine fanleaf nepovirus is implicated in RNA2 replication and colocalizes to the replication site. *Virology.* 1999;264:25–36.
- Martelli GP. Grapevine virology highlights 2006–2009. Proceedings of the 16th International Council for the Study of Virus and Virus-like diseases of Grapevine (ICVG) Conference, Dijon, France. 2009, pp 15–23.
- Hewitt WB. Virus and virus-like diseases of the grapevine. In: Frazier NW, editor. *Virus diseases of small fruits and grapevine*. Berkeley: University of California; 1970. p. 9–28.
- Hewitt WB. From virus-like to virus diseases of grapevines: some unresolved problems including immunity and ideas for researching them. *Phytopath Mediter.* 1985;24:1–7.
- Hewitt WB, Raski DJ, Goheen AC. Nematode vector of soil-borne fanleaf virus of grapevines. *Phytopathology.* 1958;48: 586–95.
- Horvath J, Tobias I, Hunyadi K. New natural herbaceous hosts of grapevine fanleaf nepovirus. *Kerteszet Tudomány (Hort. Sci.).* 1994;26:31–2.
- Huss B, Walter B, Fuchs M. Cross-protection between arabis mosaic virus and grapevine fanleaf virus isolates in *Chenopodium quinoa*. *Annal Applied Biol.* 1989;114:45–60.
- Izadpanah K, Zaki-Aghl M, Zhang YP, Daubert SD, Rowhani A. Bermuda grass as a potential reservoir host for *Grapevine fanleaf virus*. *Plant Dis.* 2003;87:1179–82.
- Margis R, Viry M, Pinck M, Pinck L. Cloning and in vitro characterization of the grapevine fanleaf virus proteinase cistron. *Virology.* 1991;185:779–87.
- Martelli GP, Savino V. Fanleaf degeneration. In: Pearson RC, Goheen A, editors. *Compendium of grape diseases*. Minnesota: APS Press; 1990. p. 48–89.
- Nicholas KB, Nicholas HB Jr. GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author; 1997.
- Oliver JE, Vigne E, Fuchs M. Genetic structure and molecular variability of *Grapevine fanleaf virus* populations. *Virus Res.* 2010;152:30–40.
- Page RDM. Treeview—An application to display phylogenetic trees on personal computers. *Comput Appl Biosci.* 1996;124: 357–8.
- Pinck L, Fuchs M, Pinck M, Ravelonandro M, Walter B. A satellite RNA in grapevine fanleaf virus strain F13. *J Gen Virol.* 1988;69:233–9.
- Pompe-Novak M, Gutierrez-Aguirre I, Vojvoda J, Blas M, Tomažic I, Vigne E, Fuchs M, Ravnikar M, Petrovic N. Genetic variability within RNA2 of grapevine fanleaf virus. *Eur J Plant Pathol.* 2007;117:307–12.
- Raski DJ, Goheen AC, Lider LA, Meredith CP. Strategies against grapevine fanleaf virus and its nematode vector. *Plant Dis.* 1983;67:335–7.
- Ritzenthaler C, Viry M, Pinck M, Margis R, Fuchs M, Pinck L. Complete nucleotide sequence and genetic organization of grapevine fanleaf nepovirus RNA1. *J Gen Virol.* 1991;72: 2357–65.
- Ritzenthaler C, Pinck M, Pinck L. Grapevine fanleaf nepovirus P38 putative movement protein is not transiently expressed and is a stable final maturation product in vivo. *J Gen Virol.* 1995;76: 907–15.
- Rowhani A, Biardi L, Johnson R, Saldarelli P, Zhang YP, Chin J, Green M. Simplified sample preparation method and one-tube RT-PCR for grapevine viruses. Proceedings of 13th Meeting of the International Council for the Study of Viruses and Virus-like Diseases of the Grapevine (ICVG), Adelaide, Australia. 2000, p 148.
- Rowhani A, Chay C, Golino DA, Falk W. Development of a polymerase chain reaction technique for the detection of grapevine fanleaf virus in grapevine tissue. *Phytopathology.* 1993;83: 749–53.
- Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. New York: Cold Spring Laboratory Press; 2001. p. 2344.
- Sanfaçon H, Wellink J, Le Gall O, Karasev A, van der Vlugt R, Wetzel T. *Secoviridae*: a proposed family of plant viruses within the order *Picornavirales* that combines the families *Sequiviridae* and *Comoviridae*, the unassigned genera *Cheravirus* and

- Sadwavirus*, and the proposed genus *Torradovirus*. Arch Virol. 2009;154:899–07.
32. Serghini MA, Fuchs M, Pinck M, Reinbolt J, Walter B, Pinck L. RNA2 of grapevine fanleaf virus: sequence analysis and coat protein cistron location. J Gen Virol. 1990;71:1433–41.
 33. Sokhandan-Bashir NS, Delpasand Khabbazi A, Torabi E. Isolation of the gene coding for movement protein from *Grapevine fanleaf virus*. Iran J Biotech. 2009;7:258–61.
 34. Sokhandan-Bashir N, Hajizadeh M. Survey for grapevine fanleaf virus in vineyards of north-west Iran and genetic diversity of isolates in the coat protein. Austral Plant Pathol. 2007;36:1–7.
 35. Sokhandan-Bashir N, Nikkhah S, Hajizadeh M. Distinct phylogenetic positions of grapevine fanleaf virus isolates from Iran based on the movement protein gene. J Gen Plant Pathol. 2007;73:209–15.
 36. Sokhandan-Bashir N, Nourinejad Zarghani S, Hejazi MS. Genetic diversity of grapevine fanleaf virus isolates from Iran. Virus Res. 2007;128:144–8.
 37. Vigne L, Bergdoll M, Guyader S, Fuchs M. Population structure and genetic variability within isolates of grapevine fanleaf virus from a naturally infected vineyard in France: evidence for mixed infection and recombination. J Gen Virol. 2004;85:2435–45.
 38. Vuittenz A. Fanleaf of grapevine. In: Frazier NW, editor. Virus diseases of small fruits and grapevine. California: University of Berkeley; 1970. p. 217–28.
 39. Vuittenz A, Munck MC, Kuszala J. Souches de virus 'a hautes agressivites isolées de vignes atteintes de d'e.g'en'erescence infectieuse. Etud Virol Appl. 1964;5:69–78.
 40. Wetzel T, Meunier L, Jaeger U, Reustle GM, Krczal G. Complete nucleotide sequences of the RNAs 2 of German isolates of Grapevine fanleaf and Arabis mosaic nepoviruses. Virus Res. 2001;75:139–45.
 41. Wetzel T, Jarda R, Meunier L, Ghorbel A, Reustle GM, Krczal G. Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. J Virol Methods. 2002;101:63–9.
 42. Wetzel T, Fuchs M, Bobko M, Krczal G. Size and sequence variability of the Arabis mosaic virus protein 2A. Arch Virol. 2002;147:1643–53.