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Status of infection with grapevine fanleaf virus in vineyards of Iran and molecular characteristics of the isolates

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ABSTRACT

Grapevine fanleaf virus (GFLV) is widespread in vineyards around the world. Investigations on GFLV from vineyards in several provinces of Iran have provided enormous information on the virus detection, distribution, recombination and even eradication in the recent decade. Primarily double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) has been applied to survey for GFLV in Iran. Later on, an initial screening by ELISA was followed by reverse transcription polymerase chain reaction (RT-PCR). In the initial PCR assays, previously designed primers were exploited, but when sequence data of local isolates were became available newly- designed primers were used that increased efficiency of the assay. As a result, sequences of movement protein (MP), coat protein (CP) and/ or even the hypothetical protein (HP) are now known for many GFLV isolates from the northwest, northeast, southwest and central part of the country as well as full-length sequence of GFLV RNA2 in four isolates. By analyses of such sequences, it has been revealed that GFLV isolates from Iran are distinct from the isolates of other parts of the world. Green grafting method on Gerey-Dash variety was also developed for screening the grafts in large scale. However, there are still gaps in our knowledge on the virus from Iran that requires further research. The ultimate goal would be control of the virus via establishing a sanitation scheme as well as exploitation of novel gene silencing strategies in order to combat the virus, save precious local cultivars and increase their productivity. This is the first comprehensive review on status of infections with GFLV in Iran.

KEYWORDS

ELISA;
GFLV;
Iran;
RT-PCR;
Variation;
Grapevine.

INTRODUCTION

Grapevine is susceptible to 58 viruses and 5 viroids. These viruses are classified in genera belonging to eight families. There are also members of unassigned genera infecting grapevine[1, 2]. Fanleaf degeneration is one of the most important viral diseases of grapevines worldwide. The disease was

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first described by Cazalis-Allut in 1865, but it was not until 1902 when Baccarini suggested that "fanleaf" may be due to a virus, which finally was confirmed by Petri in 1929[3]. The disease is caused by three virus species of the genus *Nepovirus* in the family *Secoviridae*[4]. Among them, the most important species is *Grapevine fanleaf virus* (GFLV) that is naturally vectored by the ectoparasitic nematode, *Xiphinema index*[3]. Accordingly, GFLV is widespread around the world where grapevines are grown and the yield losses can be severe. Progressive epidemic foci develop in infested vineyards within a few years following initial detection. It is a very damaging virus, causing reduced yields due to poor berry set and increasing the susceptibility of the grapevine to biotic and abiotic stresses[5].

The GFLV genome is composed of two single stranded positive-sense RNAs (RNA1 and RNA2) which carry a covalently-linked viral protein (VPg) at their 5ì extremity and a poly(A) stretch at their 3 ends (Andert-Link C, *et al*., 2004). RNA1 encodes polyprotein P1, which is processed into ûve proteins including 1A (unknown function), $1B^{Hel}$ (probably the helicase), $1C^{VPg}$ (VPg), $1D^{Pro}$ (proteinase) and $1E^{Pol}$ (polymerase). These proteins are the only proteins required for RNA1 replication, and they function in trans to ensure RNA2 replication[6]. A putative homing protein ($2A^{HP}$), the movement protein ($2B^{MP}$) and coat protein ($2C^{CP}$) are translated from RNA2 as a P2 polyprotein (Figure 1).

Grapevine (*Vitis vinifera* L.) is an important crop in Iran with a cultivation area of 313,315 ha and production of 2,795,925 tons[8]. A wide variety of grapevine cultivars are cultivated. It is produced mainly in three distinct region of Iran, namely northwest, northeast and southern. The first report of GFLV in Iran was based on visual symptoms[9]. This review encompasses our knowledge on GFLV from vineyards in Iran. GFLV is thought to have originated from ancient Persia and then spread to the west

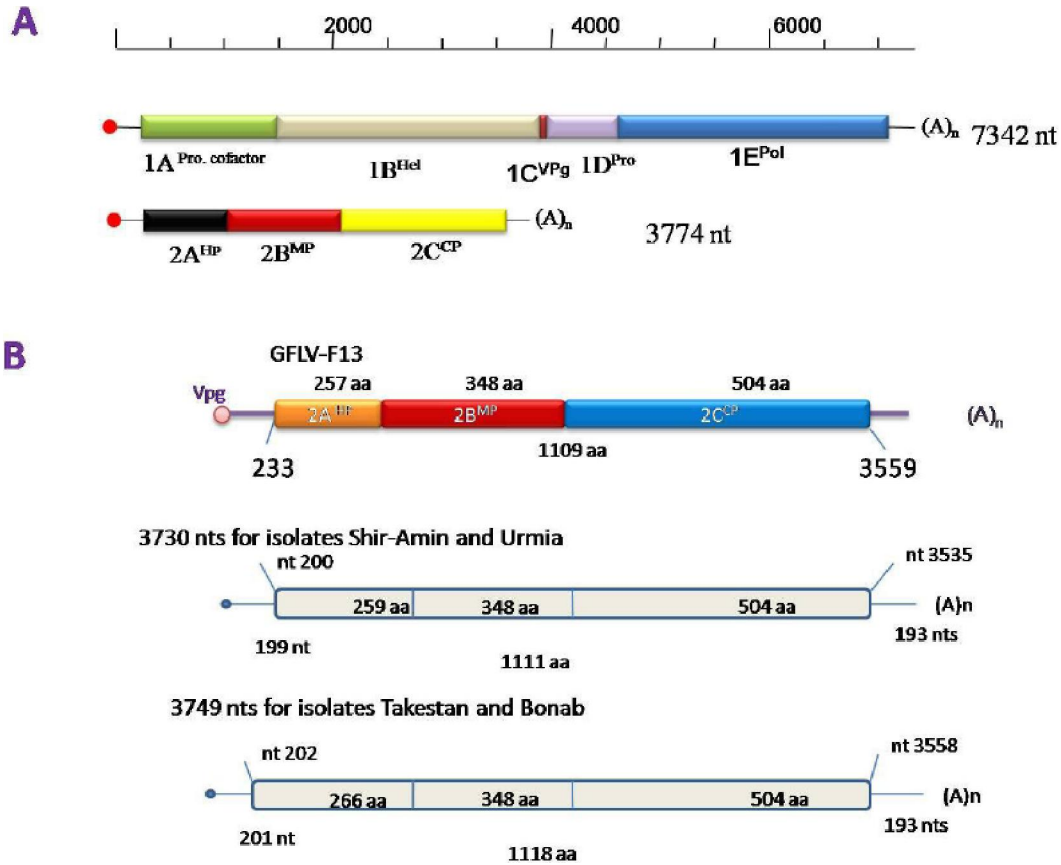

Figure 1 : Genome organization of grapevine fanleaf virus represented by that of isolate F13 (A) and the differences in the virus isolates from Iran with that of GFLV-F13[7] (B). The ruler line corresponds to nucleotide positions in the stretch of the molecules

through infected propagating material[10, 11]. However, no comprehensive review has been written on the information obtained from studying GFLV in Iran. It seems that virus-infected propagation material contributes to dissemination of the virus predominantly.

Symptoms caused by GFLV and its natural host range

Three distinct types of GFLV-associated syndromes including infectious malformation, vein banding and yellow mosaic have been described[12] and revised later by[13] as two distinct syndromes including infectious malformations (fanleaf proper) and yellow mosaic. In Iran, all kinds of GFLV symptoms as described by[1, 11, 14] have also been reported[10, 15-25]. The first report of GFLV-associated disease from Iran was solely based on symptoms[9]. The infectious malformation (fanleaf proper) was reported to include open petiolar sinus, shortening of internodes, leaf deformation, double nodes, zigzag growth of shoots, shark-toothed leaf edges, and stem or branches fasciations (Figure 2 A, B and E). The

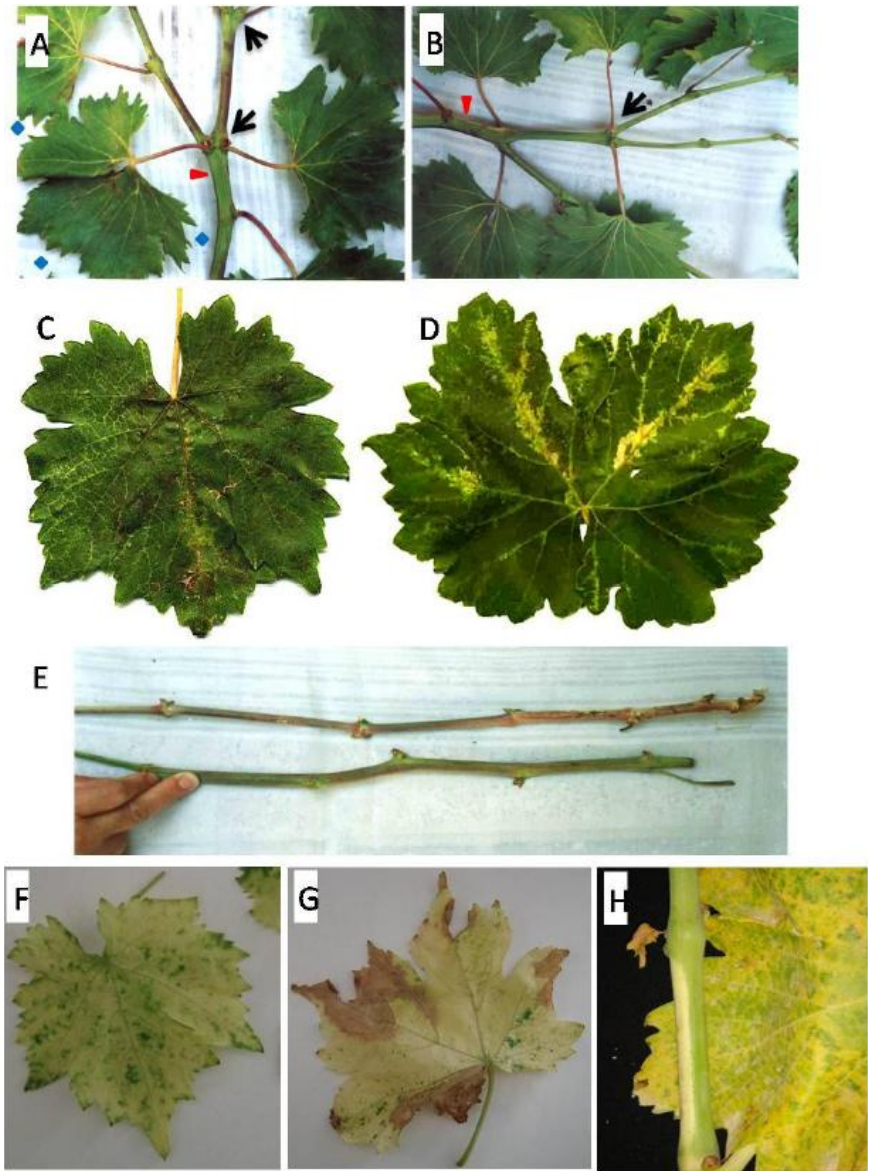

Figure 2 : Grapevine fanleaf virus symptoms on grapvines in Iran. A and B: double node (black arrows), fasciations (red triangle), open petiolar sinus, shark-toothed leaf edges (blue diamonds), C and D: vein yellowing and vein banding, E: shortened-internodes in the lower branch (infected) compared to healthy ones (upper branch), F and G: yellow mosaic syndrome on leaves starts with yellow spots or flecks, then coalesce and become necrotic, H: yellow mosaic syndrome on the branch and leaf

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symptoms reported for yellow mosaic syndrome are yellow flecks and mosaic on leaves and branches which coalesce into large yellow areas (Figure 2 F-H) and may become necrotic. Both vein yellowing and vein banding syndromes have also been reported from vineyards in Iran (Figure 2 C-D) with a difference relative to the time of the appearance as explained by[12]. Accordingly, vein banding appears in mid and late summer while in most part of the northeast region of Iran this syndrome appears mid or late April. Surveys in vineyards of Iran have shown that in southern provinces the fanleaf malformation is dominant. In our studies, we have reported this type of symptom to be prevalent in Ardabil in the northwest[20] (Figure 2A, B and E), but shortened and zigzagged internodes were seen in most areas. Therefore, although various GFLV-related symptoms are observed certain symptoms appear as predominant in each locality. For instance, in vineyards of Bonab, Malekan, Takestan (all in northwest of Iran) and Bojnoord (northeast of Iran) vein banding was prevalent while in Shir-Amin (very close to Bonab and Malekan), Hosein-Abad-e-Zahra (very close to Takestan) and Sabzehvar (close to Bojnoord) yellow mosaic and leaf distortion, shortened internodes, and stem fasciations were mostly observed.

In addition to grapevine, GFLV has also been reported from Bermuda grass (*Cynodon dactylon*) from south of Iran although there is no information on the symptom(s)[10]. Interestingly, in a recent study[26] GFLV was detected in knotweed (*Polygonum* sp.), raspberry (*Rubus ulmifolius*), Johnson grass (*Sorghum halepense*), plantain (*Plantago major*) and sweet-clover (*Melilotus* sp.). Therefore, the natural host range of the virus is not limited to *Vitis* spp. That once thought. This may suggest that other vectors are also involved in its transmission.

DISSEMINATION OF GFLV IN IRAN

Several studies dealing with GFLV in Iran have been devoted to detection of the virus whereas little attention has been paid to its dissemination. In our studies on soil samples from vineyards in the northwest of the country no vectoring nematode was isolated. This may be suggestive of dissemination through infected cuttings in that part of the country. However, the nematode vector has been recovered from soil samples of the infected vineyards in the northeast region of Iran[23]. Overall, because the grapevines are not regularly screened for virus infections it is speculated that transmission via propagating material is the main source of the virus dissemination in Iran. The ultimate goal from detection of the virus is to facilitate establishment of a sanitation scheme so that any source material can be screened for the virus before the propagation.

DETECTION OF GFLV

Efforts to optimize protocols for detection of GFLV in other countries[10, 15, 17-25, 27-31] have resulted in successful isolation of the virus variants. The relevant techniques include serological procedures (mostly ELISA) and molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) followed by sequencing. Newly established green-grafting method was also suggested for preliminary large scale screening of cuttings or nurseries by alone or in combination with RT-PCR[16].

Detection by ELISA: Enzyme-linked imunosorbent assay (ELISA) is a robust method for detection of viruses. It can be done on- site in vineyards, farms and orchards. Although RT-PCR has become popular in the detection of viruses since mid 1980s, ELISA it is still applied in large scale detection or alongside RT-PCR[17, 20-22]. Generally, double antibody sandwich (DAS)-ELISA method[32] is used in detecting GFLV; however, direct antigen coated (DAC) - ELISA method[33] has also been applied to enhance the sensitivity[22]. In DAC-ELISA the microtiter plate is not initially coated with antibody which otherwise imposes a selection on the type and number of antibody molecules being trapped in the plate. Therefore, total protein including viral protein can bind the plate with a maximal capacity (Figure 3).

In all the reports on grapevine viruses in Iran including GLFV, ELISA has been applied (Table 1). The first report is that of[25] where samples of grapevine from Fars, Kohgiluyeh and Boyer-Ahmad and West Azarbaijan provinces were surveyed for GFLV by ELISA. Accordingly, vineyards in all these provinces

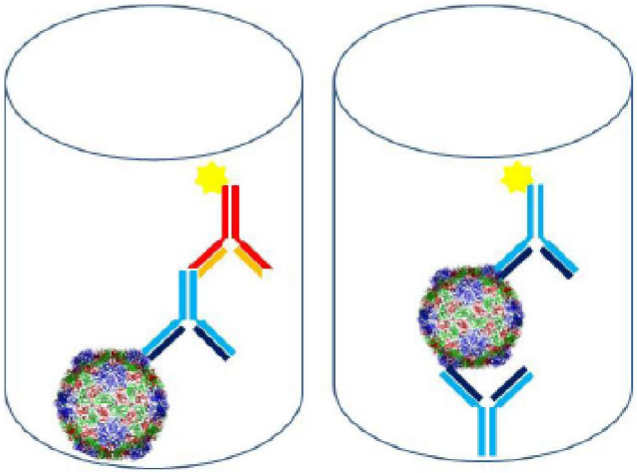

Figure 3 : A schematic representation of direct antigen coated (DAC) (left) and double antibody sandwich (DAS)-enzyme linked immunosorbent assay (ELISA) (right). Note that the conjugated antibody in DAC-ELISA is different from the detecting antibody whereas in DAS-ELISA both coating and conjugated antibodies are the same

showed infections with GFLV and percentage of the infection were 21 to 37%. As reported, the highest infected vineyards were that of Bovanat in northern Fars.

ELISA has also been applied as the only test for detection of grapevine viruses by[24]. Accordingly, vine samples from 10 main grapevine cultivation locations in Iran including 56 different vineyards in Qazvin, Zanjan, East Azarbaijan and West Azarbaijan (north-west), Hamedan, Kordestan and Kermanshah (west), Semnan (east) and Khozestan (south-west) provinces were sampled and tested. As a result, the virus infections were detected in 246 of 556 samples (44.2%).

During 2003-2007, we did three independent surveys with DAS-ELISA to check presence of GFLV in the north west of Iran and detected GFLV in 31 out of 134[20], 21 out of 86[21] and 33 out of 126[22] symptomatic samples from vineyards in East- and West- Azarbaijan and Ardabil provinces. These studies uncovered that nearly all sampled areas were infected by GFLV. In total, 84 out of 346 (24.3%) samples from symptomatic vines were infected by GFLV denoting that GFLV was not the only virus that causes the aforementioned symptoms. A survey for GFLV by the use of ELISA in vineyards of the North-East Iran revealed infection with GFLV in 305 out of 3454 (8.8% of samples from 22 vineyards) randomly collected samples[23]. The highest incidence (10.9%) was in vineyards in Bojnurd (90 out of 305 samples) whereas in vineyards of Kashmar it was 6.7%.

Later, in a report by[19], 23 out of 204 (11.3%) samples from Bavanat, Shiraz, Jahrom and Maymand from south of Iran, Urmia, Karaj and Naghadeh proved to contain GFLV. In the same year[18] 86 out of 300 (28.6%) grapevine samples from East- and West- Azarbaijan and Ardabil provinces were proved to be ELISA-positive for GFLV. A further report[17] by the use of ELISA revealed that 86 of 330 (26%) randomly collected samples from the three provinces were infected by GFLV. These reports indicated that incidence of the GFLV in the vineyards was 8.8 to 26%. In most cases ELISA detected the GFLV isolates, but there were two infected samples that did not react positively in ELISA while the virus was detected by RT-PCR in the same leaves[17]. This could be due to inefficiency of the commercial antibody in the detection of local isolates. Although it is known that GFLV titer in the grapevines drops during the summer hot season and, therefore, ELISA is not suggested for the detection

TABLE 1 : Total number of samples and % infected by GFLV as determined by ELISA in different studies in Iran

Total samples	ELISA- positive	% infected	Reference
1018	310	30	[25]
134	31	23	[20]
86	21	24	[21]
126	33	26	[22]
330	89	27	[17]
300	86	29	[18]
882	204[a]	23	[19]
3454	305	8.8	[23]

[a] **Results from ELISA and RT-PCR in this study are merged**

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in this period this problem was not accounted for those two samples which escaped ELISA. This raises the possibility that there are GFLV isolates that are not trapped by the anti- GFLV IgGs used in these studies.

Detection by RT-PCR, molecular and phylogenetic analyses: In the surveys by ELISA, GFLV was detected in a small percentage of samples. One reason could be a relatively lower sensitivity of ELISA. Therefore, RT-PCR has been applied in the research on GFLV from Iran (TABLE 2). The first report[23] has dealt with amplification of a 320 bp of the virus genomic region by a couple of previously designed primers from representative asymptomatic and symptomatic ELISA-positive samples. In another report[10] while exploring GFLV infection from Bermuda grass, a 480 bp fragment was amplified from the infected plants by the primers CP 433V and CP 912C. Later on, we adopted a whole range of optimization procedures to amplify different segments of the virus RNA2 from isolates from the northwest region. The primer combinations were the crucial part of such optimizations. Reverse transcription was accomplished by the use of oligo $d(T)_{16}$ or a GFLV-specific primer. Because of unavailability of sequences of local isolates the previously reported virus- specific primers were initially used[10] that gave a 480 bp fragment corresponding to GFLV coat protein (CP) core region from nine samples[20]. We also applied another pair of previously- designed primers[29] that resulted in amplification of an 810 bp fragment from 11 samples.

PCR products from three GFLV isolates from E. Azarbaijan (KH7–8, KH9–10 and S2–3) and two isolates from W. Azarbaijan (O59 and OB) were cloned and sequenced[20]. At the nucleotide (NT) level, replicate clones of each individual GFLV isolate were 99% identical. However, sequence variability between clones from different GFLV isolates was 5–16% and 2–7% at the NT and amino acid (AA) levels, respectively. Isolates KH9–10 and S2–3 were most closely related (95% nucleotide identity), followed by isolates O59 and OB (94% NT identity). These sequences were 84–91% (NT) and 92–96% (AA) identical to GFLV isolates from other parts of the world. In a phylogenetic analysis, isolates KH9–10 and S2–3 formed a distinct clade, so did the isolates OB, O59 and KH7–8.

A correlation was conceivable between geographical origin and the virus genotype. Accordingly, O59 and OB (W. Azarbaijan), and KH9–10 and S2–3 (E. Azarbaijan) were most closely related to each other. However, an exception was isolates KH7–8 and KH9–10 which were from the same town (Kheljan), but 14% different at the NT level. A long distance exchange of infected propagation material

TABLE 2 : Primers used in the detection of grapevine fanleaf virus from vineyards in Iran

Primer pair	Sequence (5' to 3')[a]	Size (bp)[b]	Region[c]
C primer/ V primer [25]	CCAAAGTTGGTTTCCCAAGA AACGGATTGACGTGGCTGGT	320	CP
Primer C Primer V [23, 31]	CAAGGCAAGTGTGTCCAAA TGATGCTTATAATCGGATAA	1500	CP
CP433V/ 912C [10, 19, 20]	GAACTGGCAAGCTGTCGTAGAAC GCTCATGTCTCTCTGACTTTGACC	480	CP
S2515/ A3300 [20, 27]	GGAAGAGGCCACTTCTTTCCTTGGG CCCACCAGCTTCGTGATGGTAACGC	810	CP
M0/ M4 [21, 29]	CACTCTTTGCCGAATTGCC GT(A/G/T)ATCCACTT(C/T)TCATACTG	1489	HP-MP
M2/ M4 [21, 29]	C/T)T(A/G)GATTTTAGGCTCAATGG GT(A/G/T)ATCCACTT(C/T)TCATACTG	854	MP
G2/ 3'NC [22, 29]	AGGATTGCCAGGCAAATAGG ACAAACAACACACTGTCGCC	1623 or 1629	CP
GMPF1/ GMPR1 [18]	GCGGATGGNCGNACTACYGG TCTCAYRGTCGARCTCAAWCKVGG	1044	MP
GFLV-2048/ GFLV-3559 [18]	ACGGATCCGGATTAGCTGGTAGAGGAG GTCAAGCTTCTAGACTGGGAAACTGG	1515	CP

[a] W stands for A/T, M for A/C, K for G/T, R for A/G, Y for C/T, D for A/G/T, H for A/C/T, V for A/C/G and N for A/C/G/T; [b] Size of product resulting from PCR with the related primer pair; [c] Corresponding region on GFLV RNA2 flanked by the primer pair; HP: hypothetical protein; MP: movement protein, CP: coat protein.

is thought be a possible explanation for displacement of some GFLV variants among geographically isolated populations[34].

We have also exploited the primers designed by[29]. In one study[21] RT-PCR with the primer pairs M2/M4 or M0/M4 corresponding to GFLV movement protein (MP) amplified the expected 854- and/or 1,489-bp fragment(s) from 20 and 7 samples, respectively. Four smaller and three larger PCR products were cloned and sequenced that revealed amplification of a 1,489-bp fragment from isolate La208, and a 1,495-bp fragment from isolates X300 and X400 by M0/M4. The MP region of the isolates was 1,044 nucleotides (NT) corresponding to the GFLV MP rather than to ArMV with a reported MP of 1,038 NT. An expected 854-bp fragment was amplified with M2/M4 from isolates S3-4, S1-4, K11 and KX12 corresponding to that of GFLV, whereas an 848-bp fragment has been reported from ArMV with these primers[29]. This provided further evidence that the Iranian isolates were GFLV. There were 83–86% NT and 93–94% deduced AA identities between the MPs of the sequenced isolates. Nucleotide sequence identities of 81–87 and 75–79% were found between the MP regions of these isolates and that of previously published GFLV and ArMV strains/isolates, respectively. On a consensus parsimony tree based on the NT sequences, isolates La208 and X300 remained distinct from previously reported GFLVs. No correlation could be drawn with respect to the isolates' geographical origin and their MP genotypes. Accordingly, X300 was more similar to La208 than to X400 although both X300 and X400 shared the same origin (Tabriz) but La208 was from Lahroud (300 km away). However, when the analysis was based on the AA sequences, all three GFLV isolates from Iran and the previously published GFLVs were in the same subclade. On the other hand, because the partial MP sequences of the other four Iranian isolates (K11, KX12, S1-4 and S3-4) were more similar to La208 and X300 than to X400, the majority of the cloned Iranian GFLV isolates may be distinct from the previously characterized GFLV strains/ isolates.

In another study in our lab, by the use of the primer combination G2/3'NC[29] an expected ~1620 bp DNA fragment covering full CP region, except 34 NTs of 5' end, was amplified from all the tested samples. PCR products from isolates B5, S1 and SH3 were cloned and the NT sequences of three clones from each isolate were determined. The sequences showed that a 1623 bp fragment from isolate S1, and 1629 bp from B5 and SH3 were amplified which covered 1481 NTs of the 3' proximal region of the CP gene plus 142 or 148 NTs of the 3'UTR. Sequences alignment revealed over 99% identities among clones from each isolate and 83–93% among clones from different isolates. Identities of 83–94% were found between the isolates from Iran and previously reported GFLV strains/isolates. Phylogenetic analysis based on CP sequences showed that isolates S1 and SH3 formed a distinct cluster but isolate B5 clustered with previously reported GFLV strains.

In another study[23] PCR by previously designed primers[31] gave an approximately 1500 bp fragment corresponding to GFLV CP and subsequent nucleotide sequence analysis of five representative isolates showed high identities (98.7-100%) between them. When compared with the previously reported sequences they found GFLV-USA (AF304014) as the closest (83.7-83.9%) to these GFLV isolates.

Recently, sequences of near full length RNA2 of four isolates from Iran, i.e. Shir-Amin (East Azarbaijan), Urmia (West Azarbaijan), Bonab (East Azarbaijan) and Takestan (Qazvin) (accession numbers JQ071374 to JQ071377) were determined[15]. Accordingly, amplification of the RNA2 was carried out by using 5'-NC/M4 and GFLV2048F/3'NC primer pairs which amplified 2.2 and 1.65 Kbp segments of the GFLV RNA2, respectively, covering the partial 5'- non coding region, entire $2A^{HP}$ and $2B^{MP}$, and the $2C^{CP}$ with a partial segments from 3'-non-coding region[15]. Application of one step RT-PCR with the use of different RT-PCR kits were unsuccessful, but the use of 2-step RT-PCR procedure, high quality template RNA, and lower temperature ramp (2°C/sec) resulted in amplification of 2.2 kbp of the GFLV RNA2 in all ELISA positive samples (Nourinejhad-Zarghani et al., unpublished data). RNA2s of the Shir-Amin and Urmia isolates was 3730 NT in length and 3749 NT for the Takestan and Bonab isolates, excluding the poly (A) tail. The latter isolates harbored the longest $2A^{HP}$ gene among

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the reported GFLV isolates which resulted in increasing the length of the RNA2 in these two isolates. The previously reported putative Cys/Ala and Arg/Gly proteolytic cleavage sites were also found in these isolates whereby P2 is broken down to smaller proteins. Identities of 89–97.6% NT were determined between near full-length RNA2 of the Iranian isolates whereas previously 8.3–84.8% identities were estimated for the other Iranian isolates. GFLV-F13 was the closest isolate to the Iranian isolates at the NT level. At the AA level, there were 90.9–97.9% identities among P2 of the Iranian isolates, whereas 86.3–92.7% between the Iranian isolates and previously reported isolates. The isolate WAPN173 (American isolate) was the closest to the Iranian isolates. When compared with other isolates of GFLV whose ORF2 sequence is available, identity levels of 77.5% and 88.3% were found for the $2B^{MP}$ gene, and at least 83.6% and 92% for the $2C^{CP}$ gene at the NT and AA levels, respectively.

Studies by The Use Of Newly Designed Primers: New primers were designed after sequences of local isolates were determined that expectedly enhanced efficiency of the PCRs [16-18]. By the primers GMPF1 and GMPR1 corresponding to the GFLV MP, the full length MP gene (1044 bp) was amplified from 41 of the 86 ELISA-positive samples. Sequence analyses of seven PCR products revealed up to 17 and 8% divergence between the Iran isolates at NT and deduced AA sequence, respectively. On a NT-based parsimonious tree, isolates from Iran stood distinct suggesting independent evolution of GFLV in this region. Very recently, even better results were achieved by the use of inosine as the wobble base in these primers instead of the ambiguous nucleotides so that less non specific bands were amplified (Nourinejhad-Zarghani et al., unpublished data).

In a separate study we designed a couple of primers, GFLV-2048 and GFLV-3559 to precisely amplify the virus full CP gene[17] and facilitate expression of the CP for antibody preparation. An expected 1515 bp fragment was obtained for 16 out of 89 isolates that were infected as shown by DAS-ELISA. No amplification was achieved from samples from W. Azarbaijan province although they were ELISA-positive. CP fragment from eight isolates were cloned and the NT sequences determined. Alignment of previously reported GFLV strains/ isolates and ArMV-S showed that new isolates were GFLV. Accordingly, there were over 99% similarities at NT level within clones from each isolate. Between clones from different isolates, the lowest NT similarity (92%) was found between KH4-5-3 and S-4-2-1 or KJ-16-2-3; the highest (98%) between KH4-5-3 and MG-28-1-3. At deduced AA level, the lowest similarity (95%) was found between KH4-5-3 and S-4-2-1. On the maximum likelihood (ML) parsimonious trees, based on NT or AA data, GFLV isolates from Iran formed a distinct cluster except for a previously reported isolate from Iran, B5 that clustered with other isolates when the analysis was based on NT sequences[17]. Interestingly, GFLVs from Iran were the only geographical isolates that forming a distinct cluster although a fraction of GFLV isolates from some other countries, particularly France, also stood distinct. There seemed to be a correlation between the geographical origin and phylogenetic positions of the isolates at large scale (world).

Availability of the sequence data for $2C^{CP}$ and $2B^{MP}$ genes of Iranian GFLV isolates allowed us to conduct a study to analyze identity of the targeted fragments amplified by the use of the reported primers sequences and then design further new primers for amplification of different genes on the RNA 2[16]. The results showed that GFLV-CP2-s and G2-3370s were the most efficient primer pairs for the detection of GFLV.

Detection with green-grafting method: There is no report of natural resistance against GFLV, but the level of susceptibility or response of grapevine varieties is different. The quickest and most typical responses occur on *Vitis rupestris*. In greenhouse chip-budding or green- grafting onto *V. rupestris* may be used as an indexing method to detect GFLV. At 22 to 24°C, three to four weeks after grafting chlorotic spots, rings and lines appear on the indexed plants (Martelli G P, 1993). In our survey occasionally we noticed that GFLV- expressed symptoms were more severe on Gerey-Dash variety than other varieties, so we used it as a rootstock for the indexing by green grafting method as explained by

Pathirana and McKenzie)2005(and Nourinejhad-Zarghani et al. (2012). Symptoms developed on leaves of the rootstock were similar to those observed on the corresponding scion. On the other hand, in order to determine whether the grapevine variety or the virus is playing the most important role in the symptom expression, different grapevine varieties were graft-inoculated with GFLV resulting in fanleaf degeneration, yellow mosaic or vein banding syndromes. Then, these infected varieties were used as scions for green- grafting onto a virus-free Iranian Gerey-Dash grapevine variety as the rootstock. The results uncovered that the type of symptoms was independent of the scion varieties as the source of virus, but was dependent on the virus itself. If the scion was infected by GFLV, two to three weeks after the grafting the same symptoms which incited on the source mother plant of scion also appeared on the leaves of Gerey-Dash as rootstock[16]. Therefore, this variety can be a candidate for the quick indexing proposes. It is obvious that the results should be compared with *V. rupestris* under the same environmental condition.

RECOMBINATION

In our study on occurrence of recombination in GFLV isolates, sequences of full length MP cDNAs which were amplified by the use of GMPF1 and GMPR1 from the virus isolates from the northwest region of Iran were determined. These sequences were aligned with counterpart genomic region of previously reported GFLVs and that of ArMV. When an alignment of 107 MP sequences was searched for recombination a total of 12 such events were detected in 34 recombinants[35]. Eight events were confirmed by significant *P*-values (<5%), further phylogenetic analyses and historical characteristics of the recombinants. Double events were evident in the Iranian isolates Kh29-5, La3-6-1, La3-6-3, LGR12, Sl1B and Sl1C. The events in Kh29-5 were overlapping and shared the same parent (X300-I1C1). Events 1, 5 and 8 each occurred in only one isolate including LGR12, NP2 or Kh29-5, respectively. We also documented recombination in other parts of the GFLV RNA 2. First report of recombination events in $2A^{HP}$ gene of isolates from Iran was established[36]. In parallel, similar recombination events were reported by Jawhar et al., (2009). It was shown that the $2A^{HP}$ gene is the most diverse region of GFLV RNA2 in the GFLV isolates from Iran[15] due to recombination. Similar events were also reported in *Arabis mosaic virus* and it was shown that the GFLV $2A^{HP}$ gene could replicate ArMV RNA2 in association with ArMV replication complex[38]. However, there is no report of recombination in $2C^{CP}$ gene of the Iranian isolates yet[15, 35]. Overall, all the Iran isolates appeared to be recombinants. That several events were parented by the indigenous isolates provided further clue as to the trueness of the detected recombination events because it is quite likely that isolates coexisting in a given region exchange their genomic segments. This also supported the speculation that GFLV has been in Iran since ancient times.

Population genetic and selection pressure: Recently genetic population parameters were estimated for Iranian isolates based on availability of sequence data for the partial or complete RNA 2 of the Iranian isolates[15, 35]. Based on these reports, the $2A^{HP}$ gene had the highest estimated genetic variation parameters of Θ_W (0.12989 ± 0.03794) and Pi (0.14139 ± 0.00989) values, denoting its higher nucleotide diversity in comparison with that of $2C^{CP}$ (0.06476 ± 0.02357)[15] and $2B^{MP}$ (0.08566 ± 0.03238) genes (Nourinejhad-Zarghani et al., unpublished data). These data also showed that $2C^{CP}$ gene is the most conserved gene of the RNA2 in the Iranian Isolates. It should be mentioned that the frequency or distribution of the insertion or deletion events was not uniform in $2A^{HP}$ gene because the core and 3' region of the gene was conserve than the 5' region of the gene[15]. Subsequently, the N-terminus region of the $2A^{HP}$ protein was shown to have less effect in virus replication while the core and C-terminus region of the protein had more effect in the replication. These results were obtained by exchanging the identical region of the ArMV-NW isolate with Shir-Amin and Bonab isolates of GFLV[38]. Pi(a)/Pi(s) ratios for the P2, $2A^{HP}$, $2B^{MP}$, and $2C^{CP}$ was less than 1 denoting purifying selection. Interestingly, for the 5' region of the $2A^{HP}$ and $2B^{MP}$ genes this ration values were >1

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in the Iranian isolates of GFLV meaning that these parts of the genome in Iranian GFLV isolates were under positive selection and probably these changes had positive effect in virus replication cycles.

ERADICATION OF GFLV

There is only one report as to the control of GFLV in Iran[19]. Accordingly, 70-90% of the plants (depending on cultivar) that were subjected to thermotherapy at 40/30 °C for 7 weeks became free of GFLV although they displayed some damage. The heat therapy combined with meristem culture was suggested to eradicate GFLV from infected plants by 100 %. There have been no significant differences between the two studied cultivars when thermotherapy and meristem culture were in combination. Nevertheless, as the data on molecular characteristics of GFLV from Iran is accumulating it is anticipated that the novel control strategies based on RNA silencing may be employed.

CONCLUSION AND FUTURE DIRECTION

There has been an explosive expansion of studies on GFLV in Iran in the past decade. The majority of such studies have focused on detection of the virus that is a prior step towards control of the virus. ELISA has been used as a primary screening procedure to identify the infected samples before submitting them to molecular analysis. PCR has been exploited in several studies initially by the use of previously designed primers, but there are also more recent works with the use of new primers based on sequences of local isolates. Phylogenetic studies revealed the distinct positions of the Iran isolates no matter what part of the virus genome is set as the basis for the analysis. This gives support to the hypothesis that the virus origin has been in Iran.

These studies also suggest that in most vineyards GFLV has spread through propagation material although in the northeastern vineyards the vectoring nematode has been reported in the transmission.

Only one research has dealt with eradication of the virus which suggests thermotherapy and meristem tip culture as the efficient treatment method.

Sanitation schemes are vital for the productivity of Iranian grapevine cultivars. There are a vast varieties of grapevine cultivated in the country; however, it is possible that because of vulnerability to GFLV some of these valuable varieties are getting extinct from the cultivations. To save such cultivars and , also, to control GFLV and prevent it from further dissemination the only practical method would be establishment of sanitation schemes in vineyards under supervision of local departments of agriculture. To achieve the sanitation, works should be done to facilitate robustness of the detection tools especially ELISA and PCR for regular screening in the vineyards. To that end, preparation of anti-GFLV antibodies prepared against recombinant CP seems to be very useful.

It is also worth suggesting implementation of novel strategies, based on RNA silencing, against GFLV in Iran. Recently, genetic manipulation by the use of hairpin constructs has provided promising result to protect grapevine against the disease.

ABBREVIATION

GFLV: Grapevine fanleaf virus, DAS-ELISA: double antibody sandwich enzyme-linked immunosorbent assay, DAC-ELISA: direct antigen coated ELISA, RT-PCR: reverse transcription polymerase chain reaction, CP; coat protein, NT: nucleotide, AA: amino acid

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