Short Communication

Isolation of the gene coding for movement protein from *Grapevine fanleaf virus*

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Absract
A pair of degenerate primers, GMPF1 and GMPR1, was designed on the basis of alignment of previously reported Grapevine fanleaf virus (GFLV) movement protein (MP) nucleotide sequences from Iran and other parts of the world. cDNA was synthesized by the use of Oligo d(T)18 from total RNA extraction from each diseased grapevine leaf sample and subjected to polymerase chain reaction (PCR) with the degenerate primers under a range of annealing temperatures from 48 to 62°C. It was revealed that 55°C gave the best result in terms of producing exactly the expected fragment (1044 bp) from as many samples as possible although accompanied by few fade non specific fragments. However, by application of “hot-start” PCR and annealing at 60°C the specific fragment was amplified from 41 out of 86 samples. This was the first amplification of the precise MP cDNA from GFLVs in Iran which is very important as to preparation of recombinant anti-GFLV MP antibody to use in studying the GFLV-grapevine interaction, and also for generating pathogen-derived resistant vines.

Keywords: Annealing; degenerate; GFLV; MP; Hot-start; PCR

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Grapevine fanleaf virus (GFLV) the cause of a severe disease in grapevines belongs to the genus *Nepovirus* in the family *Comoviridae* (International Committee of Taxonomy of Viruses, 2006). It is believed that the virus has been detected in western Asian and Mediterranean countries since the earliest cultivation of grapevines (Vuittenez, 1970). In Iran, the virus was first reported in 1989 in the northwestern region, based on symptoms and inoculations on indicator plants. It has also been detected in the southwestern vineyards of Iran by serological and molecular techniques (Zaki-aghl and Izadpanah, 2003). For the first time, the genetic diversity of the virus was studied based on the virus coat protein gene in the northwest of Iran (Bashir and Hajizadeh, 2007a). Having a worldwide distribution, it is nearly impossible to eradicate GFLV from vines that have been propagated with infected material and/ or infested with the vector *Xiphinema index* (Hewitt, 1958). Detection of GFLV, as is the case with other viruses, is a prerequisite in eliminating the pathogen. The virus genome is composed of two single-stranded, linear RNA segments with positive polarity (RNA1 and RNA2), each containing one open reading frame (ORF) and coding for a polyprotein. The RNA1-encoded polyprotein (P1) is processed into five proteins and RNA2 codes for the P2 polyprotein which is proteolytically processed into three proteins; the Movement protein (MP) is one of the proteins coded by RNA2. Both RNA1 and RNA2 of the GFLV strain F13 have been sequenced, which are 7342 and 3774 nucleotides (NTs) in length, respectively. GFLV isolates from Germany, France,

USA, Tunisia, Slovenia and Jordan varying genetically in the Coat Protein (CP) region, have been reported (Vigne *et al*., 2005, 2004a, b; Anfoka *et al*., 2004; Izadpanah *et al*., 2003; Naraghi-Arani *et al*., 2001). The purpose of the present study was to precisely amplify a region of the viral RNA that codes for the virus movement protein (MP). Newly designed degenerate primers were used to follow this goal. As the previous amplification of the MP gene from GFLV isolates in Iran had been carried out by primers, which were based on previously characterized strains of the virus such as F13 and NW, hence, the number of grapevine samples resulting in the amplification of the expected fragments formed a small percentage of the analyzed samples (Bashir *et al*., 2007).

There were two obstacles in the previous study. First, either partial MP was amplified or, in addition to the MP, the surrounding regions were also amplified. Thus, amplification of the precise MP region (1044 bp) could not be achieved. Second, because of possible mismatches in the genotype of the isolates from Iran as compared to those of strains F13 and NW, failure of amplification in the majority of samples occurred. Precise amplification of GFLV MP has implications as in detection of the virus by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) and also preparing the ground for further molecular studies by the use of the MP cDNA, for example, for MP-mediated protection and anti-GFLV IgG production. In terms of molecular detection of viruses, PCR is more sensitive than serological tests; however, primer design is a very essential step in adopting such an assay. This is because GFLV, like other viruses, exists as variants, thus making it difficult to design primers that can pick all the variants. The efficiency of such primers is determined by the number of isolates being picked up by the PCR assay. In this study a set of degenerate primers appeared to be very efficient in the detection because the virus isolates were picked up in 41 of the 86 infected vines.

Grapevine leaves from 300 grapevines showing GFLV-incited symptoms (Raski *et al*., 1983) such as mosaic, vein banding, open petiole leaf, mottling, leaf deformation and fanleaf were collected from vineyards in the three northwestern provinces of Iran including East and West Azerbaijan and Ardebil during the spring and summer 2007. The samples were first subjected to screening by the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977) in order to identify the infected samples. This was carried out because similar symptoms can be caused by different virus species. Accordingly, 0.5 g of leaf tissue from leaves of each vine was macerated and tested by ELISA with a 1:200 dilution of rabbit anti-GFLV IgG (Loewe, Germany). Samples with absorbance values greater than or equal to three times the average of negative samples were considered positive (infected). As a result**,** GFLV was detected in 86 samples coming from all the three provinces.

Total RNA from 100 mg leaf tissue from each of the 86 grapevines including ELISA positive, ELISA negative and untested samples (samples that showed typical symptoms) was extracted according to Rowhani *et al*. (1993) and dissolved in 30 µl of sterile deionized water. Reverse transcription (RT) with oligo d $(T)_{18}$ was performed as described in Sokhandan Bashir *et al*. (2006). PCR was performed using a reaction mixture (12.5 µl) containing 2.5 µl of cDNA, 10X PCR buffer (1.25 µl), 50 mM $MgCl_2$ (0.4 µl), 0.3U of *Taq* DNA polymerase (0.0625 µl) and 20 pmol of each primer. One set of two primers designed and used successfully in this study included GMPF1 [5´-GCG-GATGGNCGNACTACYGG-3´] and GMPR1 [5´-TCT-CAYRGTCGARCTCAAWC KVGG-3´], which correspond to nucleotides 1004-1023 (GMPF1) and 2023-2047 (GMPR1) of GFLV RNA2, respectively. In these

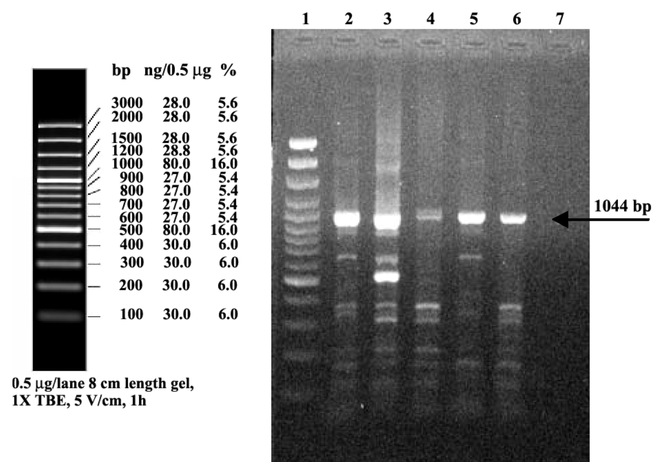

Figure 1. Electrophoresis on 1.2% (w/v) agarose of representative DNA fragments resulting from RT-PCR with the primer set GMPF1/GMPR1, corresponding to the *Grapevine fanleaf virus* movement protein (MP), from grapevine samples in the northwest region of Iran. The first lane was loaded with the 100 bp DNA ladder plus. Sample 7 is a negative control (healthy grapevine). Samples 2-6 are Kh-12, La-3, Fa-8, La-13, and Kj-18, *respectively. *Kh: Khalatpooshan (East Azerbaijan), La: Lahroud (Ardebil), Fa: Fajrabad (Ardebil), Kj: Kheldjan (East Azerbaijan).

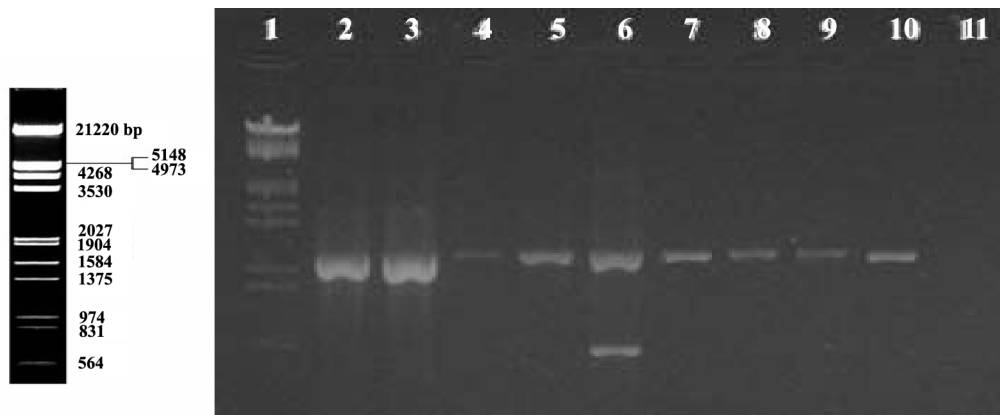

Figure 2. Electrophoresis on 1.2% agarose of products resulting from "Hot-start" PCR following reverse transcription. Lambda DNA restricted by *Eco*RI + *Hind*III was used as a marker (Lane 1). Lane 2 is a positive control (a previously cloned isolate of GFLV); lanes 3-10 are the isolates Kh-4, La-3, S-32, Kh-12, Kj-18, Kh-6 M-37 and B-10 *respectively. Lane 11 is a negative control. *Kh: Khalatpooshan, La: Lahroud, S: Sardroud (East Azerbaijan), Kj: Kheldjan, M: Malekan (East Azerbaijan), B: Bonab (East Azerbaijan).

primer sequences, 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. PCR products were fractionated on 1.2% (w/v) agarose and documented as described by Sambrook and Russell (2001).

In PCR with the primer set GMPF1/GMPR1, an initial denaturing was performed at 94ºC for 90 s followed by 35 cycles of 94 ºC for 30 s, annealing (at 48, 50, 55, 58, 60 or 62 ºC) for 30 s, extension at 72ºC for 80 s and a final polymerization cycle at 72ºC for 10 min. As a result, the expected 1044 bp DNA fragment was amplified. PCRs with different annealing temperatures were performed on different subsets of samples due to limited amounts of extracted RNA samples. Accordingly, 86 samples were tested by PCR with different annealing temperatures. The use of 55ºC as the annealing temperature resulted in amplification of the 1044 bp fragment from 30 out of 70 samples and non-specific fragments were fade (Fig. 1). Therefore, the best thermal profile for PCR with GMPF1/GMPR1 consisted of initial denaturation at 94ºC for 90 s followed by 35 cycles at 94ºC for 30 s, 55ºC for 30 s, 72ºC for 80 s and 1 cycle of 72ºC for 10 min.

In order to eliminate non-specific amplifications achieved in the aforementioned standard-PCR, "Hot-start" PCR (Chou *et al.*, 1992) was applied. Accordingly, a preliminary heating of PCR reaction mixtures (without primers) at 80ºC for 5 min was performed. The primers were then added and followed by a thermal profile incubation of 1 cycle at 94ºC for 2 min, 35 cycles of 94ºC for 1min, 60ºC for 30 s and 72 ºC for 65 s. Finally, polymerization was completed at 72ºC for 10 min. As a result, very sharp expected fragments were obtained in the absence of non-specific bands (Fig. 2). Forty one samples which gave amplifications of the expected (1044 bp) fragments in the standard PCR (as described above) produced the same fragment in the "Hot-start" PCR in absence of non-specific amplifications.

In this study, amplification of the complete MP gene from grapevine samples was carried out for the first time in Iran, being useful for genetic variation studies of GFLV at its MP level because the amplified fragments can be cloned and sequenced. Moreover, having the complete MP gene amplified, it paves the way for further studies, such as examination of potential transgenic resistance against GFLV based on MP-mediated protection (Gallitelli and Accotto, 2001).

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