TECHNICAL SHEET No. 7

Virus Detection: *Grapevine fanleaf virus* (GFLV)

Methods: RT-PCR, and IC-RT-PCR

General

Virus detected: GFLV from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription IC-RT-PCR.

Developed by

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Goals

Sensitive methods for GFLV detection.

Introduction

Degeneration is a disease in grapevines caused by Nepoviruses. This disease is spread in many grapevine-growing areas of the world. Incidence of GFLV in Palestine is about 2% (1). European Nepoviruses, referred to as fanleaf, include distorting strains that induce malformation of leaves and canes, and chromogenic strains, which cause a yellow mosaic in the plant leaves (2). A well-known example of such viruses is Grapevine fanleaf (GFLV). The American Nepoviruses induce responses in grapevines that vary depending on the grapevine species and climatic condition. Grapevine decline, stunted growth, and low yield are the major symptoms of this disease (1). European and American Nepoviruses are both transmitted for long distance by infected propagating material and for short distance by nematodes (1).

Grapevine fanleaf virus is a single-stranded RNA virus belonging to the genus Nepovirus. Fanleaf disease was first reported in *Vitis vinifera*; from Austria; by Rathay in 1883 (3). The symptoms of this disease may vary according to different host species. For example, in *Vitis* spp. symptoms include green or yellow systemic mosaic, rings, line patterns and flecks, and leaf and nodal malformation. In other susceptible host species such as *Nicotiana clevelandii* the symptoms are systemic mottling and stunting. The virus is transmitted by a vector; a nematode; *Xiphinema index* and *X. italiae* (3). Virus does not require a helper virus for vector transmission. It could also be transmitted by mechanical inoculation or by grafting. The virus is neither transmitted by contact between plants; nor transmitted by pollen (although found in pollen of *Vitis*). Serology is the best tests used to identify fanleaf virus. Seedlings or rooted cuttings of *V. vinifera* and *V. rupestris* are good bait plants in studies with nematode vectors.

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set were used to detect GFLV in the tested samples:

Primers

1. RNA polymerase coding gene, 749 bp of RNA1.
H27: 5 TTATTTGCACGCATCGGATGCGC 3
C28: 5 CGACATCAGAGAGTTCACCTAAGCC 3

2. Coat protein coding gene, 605 bp of RNA2
H: 5 GTGAGAGGATTAGCTGGTAGAGG 3
C: 5 AGCACTCCTAAGGGCCGTGACC 3

RNA extraction from grapevine stems and petioles for RT-PCR

1. Cut the sample (0.5-0.7 g of the stems or petioles) into pieces with a scalpel and grind in liquid nitrogen with a mortar.
2. Add 5 volumes times the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudpmax till you get a green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80ºC till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1M DTT, dH_2O as requied).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H_2O).
13. Incubate at 37ºC for 1 h.
14. Store at –20ºC till used in the PCR.

IC-RT-PCR

Coating

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 ml in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extraction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and mortar in the presence of carborundum.
3. Collect the homogenate and centrifuge at 10,000 rpm for 10 min at 4ºC.

4. Take the supernatant into a new tube.
5. At the end of the coating period, deposit 100 µl of the extract (supernatant) in each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, 1% Triton X-100, dH_2O as required) and heat at 65ºC for a few minutes.
7. Wash the plate 3 times with PBST.
8. Add 20 µl of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
9. Put the plate on ice and distrupt the virus particles by 5-10 sec by pipetting liquid in each well, then transfer the well content to a microfuge tube.
10. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
11. Add 5 µl of dH_2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
12. Incubate the tubes at 37ºC for 1 h.
13. Store at –20ºC untill used for the PCR.

PCR conditions:

5 µl of the cDNA, 0.2 mM dNTPs, 1X Polymerase buffer including 2.5 mM $MgCl_2$, 100 ng of each primer, and 1 unit of the *Taq* DNA polymerase. Complete to 50 µl with dH_2O.

PCR cycles:

1. For RT-PCR

(Hot start): 94ºC for 5 min then 5 cycles of 94ºC for 45 sec, 40ºC for 1 min, and 72ºC for 2 min, then 30 cycles of 94ºC for 45 sec, 45ºC for 1 min, and 72ºC for 2 min. A final step of 72ºC for 10 min.

2. For IC-RT-PCR

(Hot start): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 40ºC for 1 min, and 72ºC for 1.5min. 30 cycles of 94ºC for 50 sec, 45ºC for 1 min, and 72ºC for 1.5 min. A final step of 72ºC for 10 min.

Results

RT-PCR detection of GFLV

Figure 1. (-) : negative control, M: 1 kb ladder, 1: leaf extract detected using the GFLV coat protein primer, 2: stem extract detected using GFLV coat protein primer, 1a: leaf extract detected using GFLV polymerase primer, 2a: stem extract detected using GFLV polymerase primer, 3 and 4: not related to the subject. The color of the GFLV leaf extract used for the RT-PCR was yellow, hence it might be the reason for the negative results from some of symptomatic samples.

IC-RT-PCR for GFLV

Figure 2. M: 1 kb ladder, 1 and 2: samples, (-): negative control.
Coat protien primers were used.

Discussion

There was no difference between the detection of GFLV using both methods, when the coat protein primers were used. On the other hand, the polymerase primer pair failed to detect the virus in RT-PCR reactions.

References

1. Ra'ed Al-Kowni. (1997) Institute agronomique Mediterraneen De Bari. M. Sc. Thesis
2. Hadidi, A., Khetarpal, R. and Koganezawa, H. (1998) Plant Virus Disease Control. Chapter: 20. APS Press. The American Phytopathological Society, St. Paul, MN, USA.
3. http://life.anu.edu.au/viruses/Ictv/