TECHNICAL SHEET No. 9

Virus Detection: *Grapevine Leafroll Virus 1,3* (GLRV-1,3)

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

General

Virus detected: GLRV-1,3 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

To develop sensitive methods for GLRV-1,3 detection.

Introduction

Grapevine leafroll virus is a widespread disease caused by Closteriviruses. Seven viruses of this genus have been detected in grapevines with leafroll symptoms (1). These viruses are called Grapevine leafroll associated viruses 1-7 (GLRaV 1-7). Leafroll is one of the most spread viral diseases in the Palestinian viticulture. Leafroll viruses are transmitted by infected propagating material and some of them by mealybugs (2). Since leafroll associated viruses multiply in the grapevine phloem, the leaves of infected vines are thicker than normal with discolored margins rolled downwards.

Grapevine leafroll associated viruses 1-7, which belong to the genus Closterovirus, consist of a filamentous particle and a single-stranded RNA genome. The disease caused by these viruses is a widely distributed and affecting grapevine in all viticulture. It causes significant reduction in yield and quality of the crop. Preliminary survey in Palestine showed that about 30% of the trees are infected by leafroll viruses (2). The major symptoms include down rolling of leaves and inter-veinal chlorosis. Leaf rolling begins at the base of the cane and spreads to younger leaves during midsummer. Discoloration of leaves (reddish-purple in red-fruited, see figure 3, and yellowish in white-fruited cultivars) is conspicuous in late summer and early autumn in intolerant cultivars (3). Leafroll is symptomless in some American *Vitis* spp. and hybrids (3). These viruses are transmitted by mealy bugs (some viruses) and grafting (1).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set was used to detect GLRV 1,3 in the tested samples:

Primers

1. Designed by Hadidi and Minafra for part of the viral RNA polymerase gene, amplified fragment of 340 bp:

C547 (22 bases): 5 ATTAACTTGACGGATGGCACGC 3
H229 (20 bases): 5 ATAAGCATTCGGGATGGACC 3

2. We use another primer set which was design by the Tunisian MERC team, M. Marrakchi and Hatem Fakhfakh and associates at University of Tunisia, for the coat protein gene. Amplified fragment of 945 bp.

C50 (27 bases): 5 CGTAGGCTACTTCTTTTGCAATAGTTGG 3
H49 (25 bases): 5 ATGGCATTTGAACTGAAATTAGGGC 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 place in liquid nitrogen and grind with mortar.
2. Add 5 volumes 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 until you get 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 used.
6. Dilute the extract to 40% in distilled water containing 1% 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, 0.1 M DTT, qsp with dH_2O).
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 H2O).
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 µl in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extaction: (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 carborudpmax.
3. Collect the homogenate and centrifuge at 10000 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) per 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, qsp with dH2O) and heat at 65ºC for a few min.
8. Wash the plate 3 times with PBST.
9. Add 20 µl of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
10. Put the plate on ice and release the virus by 5-10 sec pipetting liquid in each well, then transfer the well contents to a microfuge tube.
11. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
12. Add 5µl of dH_2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
13. Incubate the tubes at 37ºC for 1 h.
14. Store at –20ºC till 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

1. Conditions using the Polymerase primer showed faint bands: (Hot start): 94ºC for 5 min, then 10 cycles of 94ºC for 50 sec, 48ºC for 50 sec, and 72ºC for 1 min. 25 cycles of 94ºC for 50 sec, 54ºC for 50 sec, and 72ºC for 1 min. A final step of 72ºC for 5 min.
2. Conditions using the Polymerase primer producing non-specific products together with the specific one: (Hot start) 5 min at 94ºC, then 35 cycles of 40 sec at 94ºC, 50 sec at 48ºC, and 1 min at 72ºC. A final step of 5 min at 72ºC. The annealing temperature of the primer is 54ºC, so if the temperature is reduced to 48ºC by mistake, many non-specific bands together with the specific one are present.

2. For IC-RT-PCR

1. Using the coat protein primer: (Hot start) 94ºC for 5 min, then 15 cycles of 94ºC for 50 sec, 45ºC for 1 min, and 72ºC for 1 min; 20 cycles of 94ºC for 50 sec, 48ºC for 1 min, and 72ºC for 1 min. A final step of 72ºC for 10 min.
2. Using the Polymerase or the coat protein primer: (Hot start) 94ºC for 5 min, then 15 cycles of 94ºC for 50 sec, 54ºC for 1 min, and 72ºC for 1 min. 20 cycles of 94ºC for 50 sec, 46ºC for 70 sec, and 72ºC for 1 min. A final step of 72ºC for 10 min.

Results:

RT-PCR detection of GLRV1,3

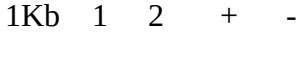

Figure 1. L: Lambda ladder, 1,2: the sample amplified with polymerase primers. +: positive control (340 bp), -: negative control using the polymerase primer.

IC-RT-PCR for GLRV

Figure 2: IC-RT-PCR of a grapevine sample: 1-Kb: ladder, 1: the sample amplified using polymerase primer (340 bp), 2: the same sample amplified using the coat protein primer (about 940 bp).

Comments

The virus was detected by both sets of primers. However, the coat protein primer was better, since it didn't produce non-specific bands. Both the RT-PCR and the IC-RT-PCR were successfully applied for the detection of GLRV 1,3 in total RNA extracts of the tested samples.

References

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