

TECHNICAL SHEET No. 30

General Detection of Viroids

Methods: RT-PCR, Electrophoresis

General

Viroids detected: All viroids or viroids-like pathogens from infected plants (citrus, grapevine, stone fruits)

General method: Electrophoresis, RT-PCR

Developed by

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Goals

To develop sensitive and specific method for viroid detection based electrophoresis and RT-PCR amplification.

Introduction

Yield and quality losses are caused by viroids in vegetable, horticultural, field and ornamental crops. The *Potato spindle tuber viroid* (PSTVd) could cause 17 to 65% yield losses depending on the viroid strain and potato cultivar. The losses could reach 100% by the third generation of growth. Up to 1982, in the Philippines, over 30 million coconut trees had been killed by cadang-cadang disease.

Viroids are composed only of a circular, single-stranded RNA molecule of 240-400 nucleotides. Unlike viruses, they do not code for any protein and therefore must rely on host enzymes for their replication. In general, it is believed that they elicit their pathogenic effects by direct interaction between either the viroid RNA itself, or other viroid-specific RNAs generated in the course of the infection, and one or more cellular targets (Flores, 2001). Viroids can be classified into two separate families based on comparative sequence analyses and whether or not they possess a central conserved region (CCR) (Flores et al., 2004). There are currently only four members in the *avsunviroidae* family, whose type member is *Avocado sunblotch viroid* (ASBVd), in which both plus (+) and minus (-) RNAs have the ability to self-cleave their RNA multimers. All other viroids identified to date fall into the *pospiviroidae* family, whose type member is PSTVd. The latter family possesses a highly conserved central region (CCR), and has not been shown to be capable of any specific self-cleavage reaction. Viroids are transmitted mechanically. This mechanism of transmission is wide-spread among plant pathogens where the infectious agent is transmitted following contact between an infected plant (or infested tool, carrier insect) and a wounded, non-infected plant.

Materials and Methods

Viroid RNA isolation and fractionation from plant tissue with phenol

1. Homogenate 10 g of plant tissue in a Polytron for 3 min with:
 - 40 ml of water-saturated phenol (neutralized)
 - 10 ml of Tris-HCl 0.2 M pH 8.9
 - 2.5 ml EDTA 0.1 M pH 7.0
 - 2.5 ml SDS 5%
 - 1.25 ml β -mercaptoethanol
2. Centrifuge the mixture for 15 min at 8,000 rpm.
3. Remove aqueous phase and re-extract with 0.5 volumes of water-saturated phenol.
4. Centrifuge the mixture for 15 min at 8,000 rpm.
5. Remove aqueous phase, bring it to a final volume 20 ml with water, and add:
 - 3.7 ml 10X STE (1 M NaCl, 0.5 M Tris, 10 mM EDTA, pH 7 with HCl)
 - 13.4 ml of EtOH little by little while shaking
 - 1.25 g of non-ionic cellulose (CF-11, Whatman)Shake it at room temperature for at least 1 h
6. Centrifuge the mixture for 5 min at 3,000 rpm and discard the supernatant.
7. Wash the pellet three times with 30 ml of 35% EtOH in 1X STE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.2 [adjust pH with HCl]); each time the mixture is centrifuged for 5 min at 3,000 rpm at room temperature. (To prepare 1 liter 10X STE: 60.57 g Tris, 58.44 g NaCl and 3.72 g EDTA, and bring it to pH 7.2 with HCl).
8. To elute the nucleic acids, resuspend the CF-11 pellet in 3.3 ml 1X STE, centrifuge the mixture for 5 min at 3,000 at room temperature, and recover the supernatant. Repeat this step two more times.
9. Add to the combined supernatant 2.5 vol of EtOH and mix. Keep the sample for at least 2 h at -20 °C.
10. Centrifuge the sample at 8,000 rpm for 30 min and discard the supernatant.
11. Air-dry the pellet for 30 min, and resuspend it in water. Add 0.25 ml of water per 10 g of fresh tissue.

Viroid RNA isolation and fractionation from plant tissue without phenol

1. Homogenate the plant tissue (20 g) in a Polytron for 3 min in the presence of extraction buffer (50 mM EDTA pH 7.0, 500 mM NaCl, 100 mM Tris-HCl pH 8.0, 10 mM β -mercaptoethanol). Normally, 5 ml of extraction buffer per gram of fresh tissue should be used.
2. Add 5 ml of 20% SDS followed by an incubation of 20 min at 65°C.
3. Add 25 ml of sodium acetate 3 M, pH 5.5 and incubate the suspension at 4°C for 20 min.
4. Centrifuge the mixture for 15 min at 8,000 rpm.
5. Collect the supernatant carefully (125 ml) and transfer it to a centrifuge tube.
6. Add 23 ml of 10X STE (1 M NaCl, 0.5 M Tris, 10 mM EDTA, pH 7, adjusted with HCl).
7. Add 85 ml of EtOH little by little while shaking.
8. Add 2.5 g of non-ionic cellulose (CF-11, Whatman), and shake it at room temperature for at least 1 h.
9. Centrifuge the mixture for 5 min at 3,000 rpm and discard the supernatant.
10. Wash the pellet three times with 30 ml of 35% EtOH in 1X STE % (10 mM Tris, 100 mM NaCl, 1 mM EDTA pH 7.2 con HCl); each time the mixture is centrifuged for 5

- min at 3000 rpm at room temperature. (To prepare 1 liter 10X STE: 60.57 g Tris, 58.44 g NaCl and 3.72 g EDTA, and bring it to pH 7.2 with HCl).
11. To elute the nucleic acids, resuspend the pellet in 3.3 ml 1X STE, centrifuge the mixture for 5 min at 3000 at room temperature, and recover the supernatant. Repeat this step two more times.
 12. Add to the combined supernatant 2.5 vol of EtOH and mix. Keep the sample for at least 2 h at -20°C.
 13. Centrifuge the sample at 8000 rpm for 30 min and discard the supernatant.
 14. Air-dry the pellet for 30 min, and resuspend it in water. Add 0.5 ml of water per 20 g of fresh tissue.

Treatment with methoxyethanol to remove polysaccharides (optional)

1. Mix on ice in a 15 ml Corex tube:
 - 1 vol of extract
 - 1 vol of K_2HPO_4 2.5 M, pH 8.0 (for 2 ml of extract, add 2 ml of K_2HPO_4 2.5 M and 40 μ l of H_3PO_4 85%)
 - 1 vol of methoxyethanol
2. Mix by vortexing and keep on ice for 5 min.
3. Centrifuge the suspension at 3,000 rpm for 3 min. Recover the aqueous phase and transfer it to a new tube.
4. Add per volume of the aqueous phase,
 - 0.05 vol of sodium acetate 3 M, pH 5.5
 - 0.5 Volume CTAB 1% (CTAB is cetyl trimethyl ammonium bromide)
5. Mix by shaking and keep on ice for 5 min.
6. Centrifuge the mixture at 8,000 for 20 min and 4 °C.
7. Discard the supernatant and dry the pellet.
8. Resuspend the pellet in 2 ml of 1 M NaCl.
9. Add 6 ml of cold EtOH and mix.
10. Keep the sample at -20 °C for at least 2 h.
11. Centrifuge the sample at 8,000 rpm for 20 min and discard the supernatant.
12. Dry the pellet and resuspend in water (200 μ l aprox.)

Fractionation with high salt (optional)

If the extract is not clean, this extra step can be done.

1. Add to 500 μ l of extract:
 - 55 μ l 10X STE
 - 140 μ l LiCl 10 M
2. Vortex and keep the mixture overnight at 4 °C.
3. Centrifuge for 20 min at 10,000 rpm and collect the supernatant.
4. Add to the collected supernatant:
 - 70 μ l sodium acetate 3 M pH 5.5
 - 3 vol of EtOH
5. Keep the sample at -20 °C for at least 2 h.
6. Centrifuge the sample at 10,000 rpm for 20 min and discard the supernatant.
7. Dry the pellet and resuspend in water (500 μ l aprox.).

Viroid analysis by electrophoresis polyacrylamide gels (12 cm X 14 cm X 2 mm)

PAGE 5% (Non-denaturing conditions)

H ₂ O	28.6 ml
10X TAE	4.0 ml
Solution A	6.7 ml
TEMED	60.0 µl
Ammonium persulfate (10%)	0.6 ml

Load samples in 1X TAE with glycerol. Gels are run for 2.5 hours in 1X TAE at constant intensity (70 mA) (corresponding approximately to 200 V).

PAGE 5% (Denaturing conditions, 8 M urea, 1X TBE)

Urea	16.8 g
H ₂ O	8.0 ml
5X TBE	7.0 ml
Solution A	5.8 ml
TEMED	30.0 µl
Ammonium persulphate (10%)	0.5 ml

The electrophoresis buffer is 1X TBE and gels are run for 1.5 h at constant voltage (200 V), corresponding to an approximate intensity of 50 mA.

Before loading the samples, remove the undissolved urea from wells with some buffer. Add 1 mg urea/µl of sample (and 0.2 µl of a solution of xylene cyanol and bromophenol blue). Dissolve with a vortex, heat the samples at 65 °C for 10 min, cool them down in ice, and load (avoid a long time in ice to prevent urea precipitation).

PAGE 5% (Denaturing conditions, 8 M urea, 0.25X TBE)

Urea	16.8 g
H ₂ O	11.7 ml
5X TBE	1.75 ml
Solution A	5.8 ml
TEMED	30.0 µl
Ammonium persulphate (10%)	0.5 ml

The electrophoresis buffer is 0.25X TBE and gels are run for 3 h at constant intensity (17 mA), setting a maximum voltage of 350 V.

Before loading the samples, remove urea from wells with some buffer. Add 1 mg of urea/µl of sample (and 0.2 µl of a solution of xylene cyanol and bromophenol blue). Dissolve with a vortex, heat the samples at 65°C for 10 min, cool them down in ice, and load (avoid a long time in ice to prevent urea precipitation).

SOLUTIONS AND BUFFERS

SOLUTION A

Acrylamide	15 g
Bis-acrylamide	0.38 g
H ₂ O	50 ml (final volume)

Staining of Polyacrylamide gels with silver

Solution I (100 ml):

10 ml ethanol
0.5 ml acetic acid (glacial)
89.5 ml water

- 3 minutes with solution I
- 2 washes with distilled water

Solution II: AgNO₃ (0.2%)

- 15 minutes with 100 ml of Solution II
- 2 washes with distilled water

Developing

Solution III (100 ml):

3 g NaOH
96.5 ml distilled water
0.5 ml formaldehyde (37%)

Amplification of CEVd by RT-PCR

Reverse Transcription

5 µl Plant extract

2 µl (200 ng) reverse primer

heat 3 min at 95°C and directly transfer on ice for 5 min

Add: 4 µl MMLV-RT buffer 5X

2 µl dNTP (10 mM each)

2 µl DTT

1 µl MMLV (200 U), MMLV-RT (Murine molony lococyte virus-reverse transcriptase)

4 µl H₂O

Incubate for 1 h at 37°C

Polymerase chain reaction

5 µl cDNA (reverse transcriptin reaction, first strand complementary DNA)

1 µl reverse primer (100 ng) 5' CCCGGGGATCCCTGAAGGACTTC 3'

1 µl forward primer (100 ng) 5' GGAAACCTGGAGGAAGTCGAGG 3'

2 µl Taq polymerase buffer 10X

0.5 µl *Taq* DNA polymerase 2.5 U)

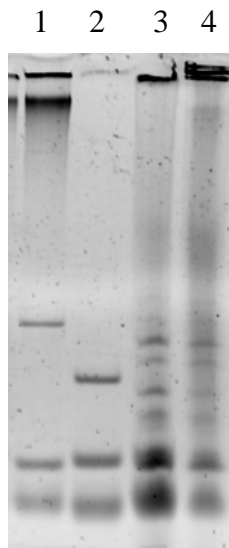
12.5 H₂O

PCR Program

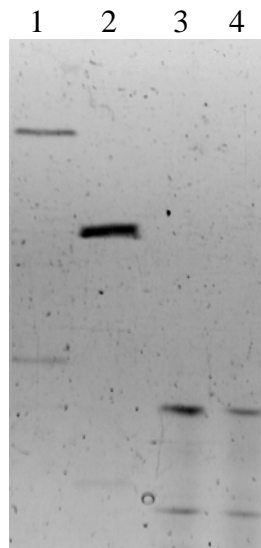
2 min at 94°C, 1 time, Denaturation 1 min at 94 °C, Hybridation 1 min at 56°C, Elongation 1 min at 72°C, repeat 35 times, then 10 min at 72 °C, final extension,1 time, and hold at 4°C or RT.

The reaction products are analyzed by 1.5% agarose gel electrophoresis.

Results



Native Gel



Denaturated gel

1. Extract from Gunyra infected with CEVd (*Citrus exocortis viroid*)
2. Extract from avocado infected with ASBVd (*Avocado sunblotch viroid*)
3. Extract from healthy Gunyra
4. Extract from healthy avocado

References

- Flores R. 2001. A naked plant-specific RNA ten-fold smaller than the smallest viral RNA: the viroid. *C R Acad Sci.*; 324: 943–52.
- Flores R., Randles J.W., Bar-Joseph M., Owens R.A., Diener T.O. 2004. Viroidae. In *Virus Taxonomy, Eighth Report of the International Committee on Taxonomy of Viruses*, ed. CM Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, A.L. Ball, pp. 1145–1159. London: Elsevier/Academic.