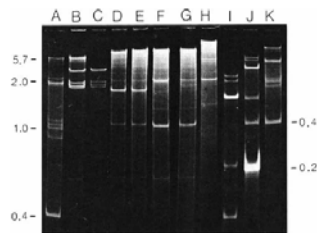


National Plant Diagnostic Network Virus Diagnostics workshop

dsRNA analysis

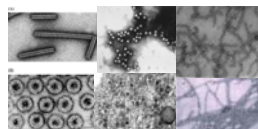


Virus Detection Methods

An early step in any approach to treat and manage viral diseases involves detection and identification of the pathogen.

Positive identification of plant viruses can be accomplished by variations or combinations of at least four different technologies:

- Plant host range & transmission (a biological assay)
- Electron microscopy (a biophysical assay)
- Serology-based assays
[e.g., Enzyme-linked immunosorbent assays]
- Nucleic acid-based analysis (a molecular approach)
 - **dsRNA analysis**
 - Polymerase chain reaction [PCR] assays
 - Macroarray and Microarray
 - "Next Generation" sequencing



How are Viruses Classified?

Particle morphology:

- Isometric, spherical
- Flexuous or rigid rods
- Bullet-shaped
- Enveloped or non-enveloped

Genome:

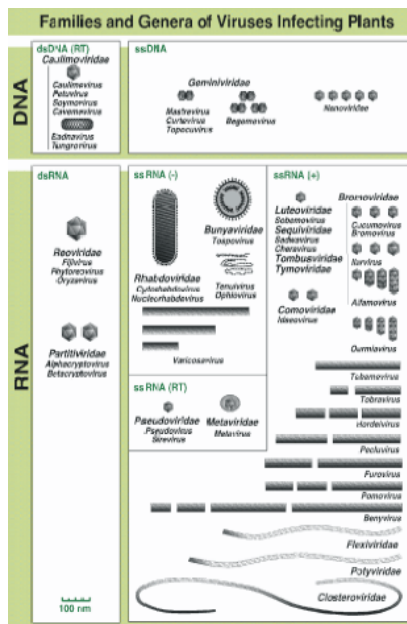
- ssRNA, dsRNA
- ssDNA, dsDNA
- Plus- or minus-sense
- Mono- or polycistronic

Host:

- Vertebrates (Animals),
- Invertebrates, Insects,
- Plants, Algae, Protoza,
- Fungi, Bacteria

Transmission:

- Airborne, Fluids, Cells,
- Vector (such as insects)



dsRNA analysis – a non-discriminatory assay

- No knowledge of the possible identity of the virus is required. No Abs, primers or probes.
 - RNA virus replication model: Number and size of dsRNA products should predict genome size and components of infecting virus.
 - The occurrence of high molecular weight dsRNA in plants is associated with the presence of dsRNA viruses or ssRNA viruses.
 - DsRNA is stable, can be extracted easily from the majority of plant species.
 - Provides an excellent tool for characterization of novel viruses that are recalcitrant to identification by other methods.
 - Healthy plants = no dsRNA?
 - Methods
 - Examples
 - Advantages and Disadvantages
- Key methods and background references:
- Valverde, Nameth, and Jordan (1990) Analysis of Double-Stranded RNA for Plant Virus Diagnosis (Special Topic). *Plant Disease* 64:255-258.
 - Dodds, Morris, and Jordan (1984) Plant Viral Double-stranded RNA. *Annual Review Phytopathology* 22:151-168.
 - Morris, Dodds, Hillman, Jordan, Lommel, and Tamaki (1983) Viral Specific dsRNA: Diagnostic Value for Plant Virus Disease Identification. *Plant Molecular Biology Reporter* 1:27-30.
 - Tzanetakis and Martin (2008) A new method for extraction of double-stranded RNA from plants. *J. Virol. Methods* 149:167-170.

dsRNA analysis - 1

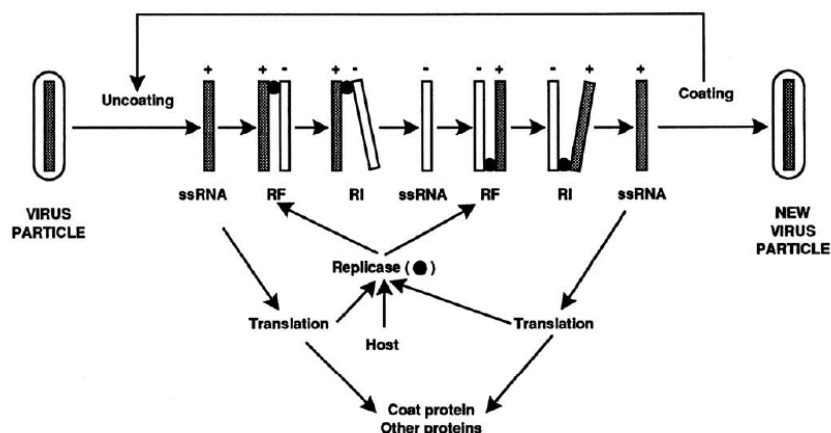


Fig. 1. Schematic representation of the proposed replication cycle of a typical single-stranded RNA (ssRNA) plant virus. Only the replication of the genomic RNA is illustrated. In some cases, however, replicative forms (RFs) of subgenomic RNAs are also produced. Double-stranded RNAs obtained from plants infected with ssRNA viruses are probably RFs. Replicative intermediate (RI) consists of dsRNA that is partially single-stranded and therefore more susceptible to RNase degradation.

From: Valverde, Nameth, and Jordan (1990)
Plant Disease 64:255-258 (Special Topic).

dsRNA analysis - 2

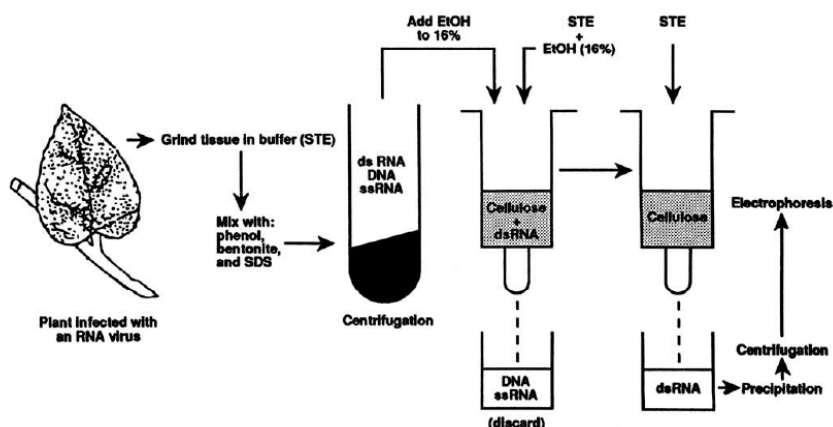
Extraction, isolation and purification of dsRNA from plant tissue.

- General Methods –
 - Original 2M/4M LiCl partitioning of total nucleic acid samples:
Salt fractionation procedures are most useful when the purpose is to separate and analyze the various nucleic acid fractions. Precipitation of dsRNA in 4M lithium chloride and solubility in 2M lithium chloride has been clearly demonstrated and was the standard procedure for isolating and purifying dsRNAs.
 - Differential nucleic acid (in presence of various % ethanol) binding to, and elution from, a cellulose-based matrix - CF-11 chromatography [Franklin, 1966; modified for plant and fungal sources by Morris and Dodds, 1979].
 - Franklin: Bind all nucleic acids at 35% EtOH, DNA and ssRNA elute at 15-18% EtOH, highly structured RNAs (dsRNA and viroids) elute at 0-5% EtOH.
 - Current: selectively bind dsRNAs at ~15-16% EtOH, elute at 0% EtOH
 - Tube PAGE gels → thin slab PAGE gels → agarose gels.
 - Enzyme treatments to confirm dsRNA
 - DNase resistant; RNase resistant in high salt; RNase susceptible in low salt.

From: Dodds, Morris, and Jordan (1984) Annual Review
Phytopathology 22:151-168 .

dsRNA analysis - 3

Schematic of basic steps in the extraction, isolation and purification of dsRNA from plant tissue using cellulose chromatography.



From: Valverde, Nameth, and Jordan (1990)
Plant Disease 64:255-258 (Special Topic).

dsRNA analysis - 4

Extraction, isolation and purification of dsRNA from plant tissue.

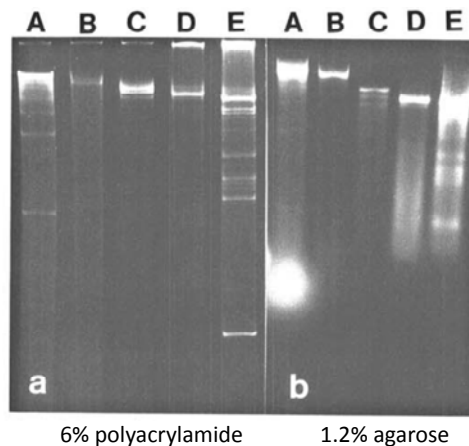
- Other Methods –
 - Columns vs. 'Batch' (smaller tissue weights and cellulose binding, washes and elution in microfuge tubes via centrifugation)
 - Attempts to extract dsRNAs without use of phenol and chloroform have been reported. However, qualitative and quantitative recovery is highly variable in most alternates and are usually host dependent.
 - Alternative cellulose-based matrix? (Whatman CF-11 now in short supply). [Sigma product under evaluation].
 - Total RNA spin-columns (e.g., Qiagen) not adequate for some virus-host interactions (too low starting tissue mass).
- Following will be examples of dsRNAs from:
 - ✓ Rod-shaped viruses
 - ✓ Spherical viruses
 - ✓ Various single virus infection
 - ✓ Various mixed infections
 - ✓ Differentiation of variation in field isolates
 - ✓ Discovery of new/novel viruses
 - ✓ Batch method and dsRNA stability
 - ✓ "Healthy plant" dsRNAs?
 - ✓ Disadvantages and Advantages

dsRNA analysis - 5

Example of dsRNAs of various sources infected with viruses with elongated particles and undivided genomes (poty-. carla-. potex-. and tobamoviruses).

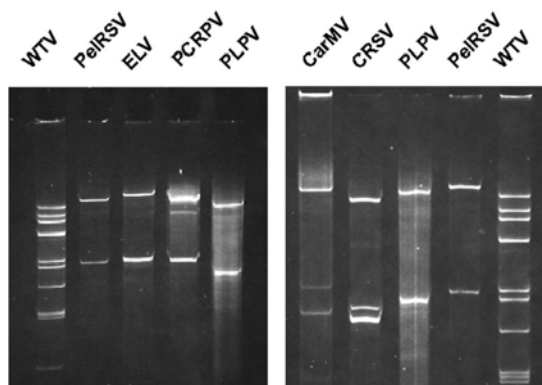
- A: Citrus tristeza virus (closterovirus)
 B: Tobacco etch virus (potyvirus)
 C: Dandelion latent virus (carlavirus)
 D: Potato virus X (potexvirus)
 E: Tobacco mosaic virus (tobamovirus)

- The size and number of the expected dsRNAs corresponding to the encapsidated ssRNA genomes (the RF) were diagnostic for each virus group analyzed.
- Other dsRNAs of smaller size than the RF were consistently obtained with most viruses.
- The presence or absence of additional dsRNAs, together with their size, were useful characteristics for differentiating particular viruses, including strains.



From: Valverde, Dodds, and Heick (1986) *Phytopathology* 76:459-465.

dsRNA analysis - 6

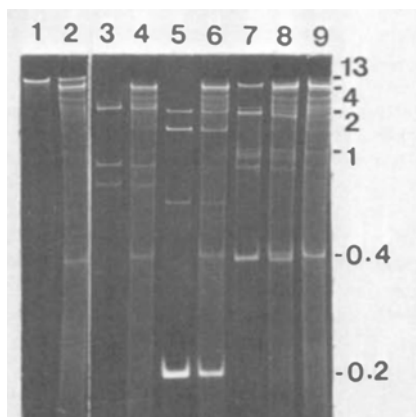


Note different dsRNA profiles of several Tombusviridae genera:

- Carmovirus (CarMV)
 - 1 genomic, 2 subgenomic
- Dianthovirus (CRSV)
 - 2 genomic (1 subgenomic?)
- Pelarspovirus, a new genus (PelRSV, ELV, PCRVP, PLPV)
 - 1 genomic, 1 subgenomic

From: Kinard and Jordan (2002) *Acta Horticulturae* 568:17-27.

dsRNA analysis - 7



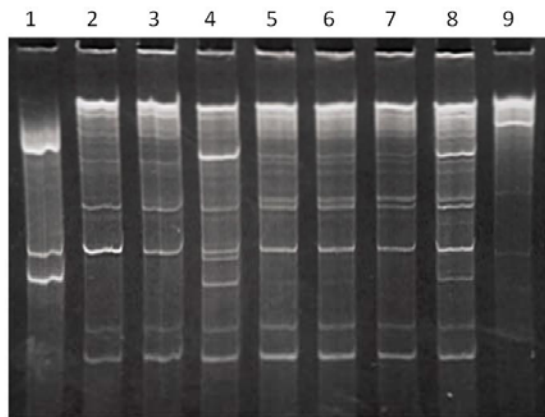
CTV TNV CMV Hm9 TMV
+RNA5 +PcV
+PsV
Plus TMV added in lanes 2, 4, 6, 8

Note different dsRNA profiles of the different viruses and ability to differentiate single infections of CTV, TNV, CMV, and TMV (lanes 1, 3, 5, 7, 9) from the mixtures (lanes 2, 4, 6, 8).

From: Dodds, Morris, and Jordan (1984) Annual Review
Phytopathology 22:151-168 .

dsRNA analysis - 8

DsRNA isolated from individual carnation plants infected with: either *Carnation necrotic fleck virus* (CNFV; lanes 2, 3, 5, 6, 7), or *Carnation mottle virus* (CarMoV; lane 1), or both (lanes 4, 8). [Lane 9 = 'Black Turtle Soup' bean endogenous dsRNAs].



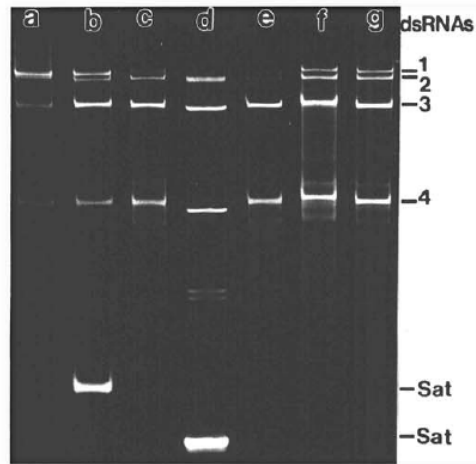
Note different dsRNA profiles of the different viruses, and ability to differentiate single infections from a mixed infection.

Note also the typical complex dsRNA pattern for closteroviruses (dsRNAs from genomic and subgenomic ssRNAs).

From: Jordan and Guaragna (unpublished).

dsRNA analysis - 9

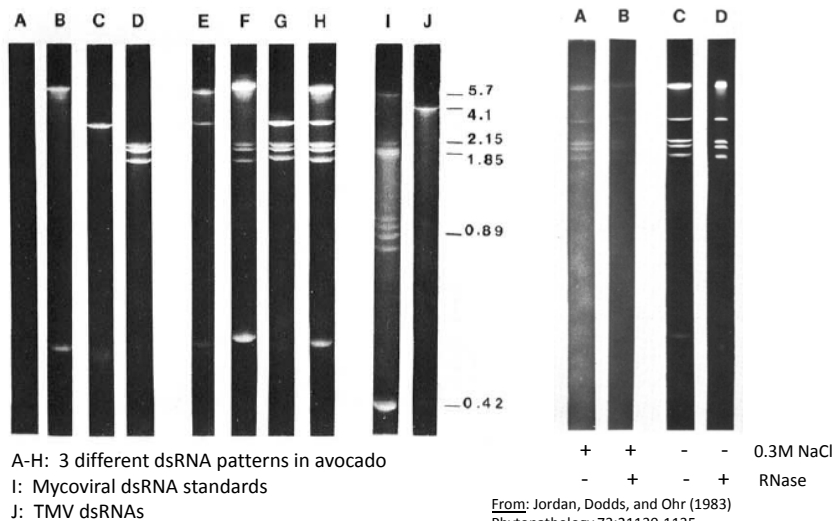
Example of dsRNAs from various strains and field isolates of *Cucumber mosaic virus* (CMV) [3 genomic ssRNAs, 1 sub-genomic ssRNA, +/- satellite RNA(s)].



From: Valverde, Nameth, and Jordan (1990)
Plant Disease 64:255-258 (Special Topic).

dsRNA analysis - 10

Example of new/unknown viral dsRNAs (3 species) from avocado. Confirmation = dsRNA via resistance to RNase in 0.3M NaCl, susceptibility in buffer w/o NaCl.



dsRNA analysis - 11

Example of dsRNAs via “mini-batch” method [0.1-0.5g; CF-11 in microfuge (not columns)] from various sources after 4hr (A-G, I) or 2 hr (H, J, K) PAGE electrophoresis.

B-C: Avocado viral dsRNAs

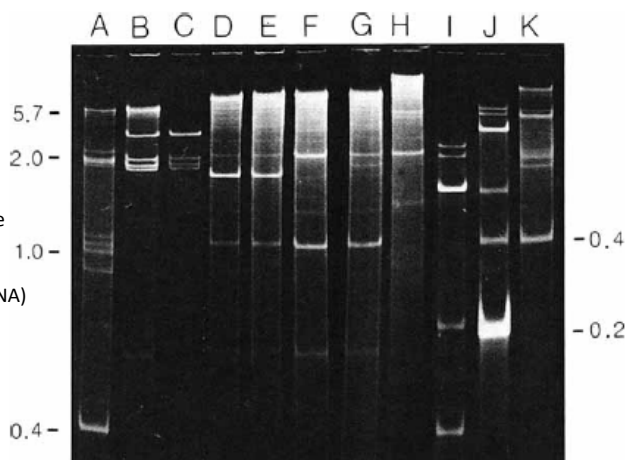
D-H: CTV in citrus dsRNAs

[D: dsRNA stored 20-day RT]

[E: dsRNAs from 8Kxg particulate fraction from VP]

I-J: CMV dsRNAs (+ Satellite dsRNA)

A, K: Mycovirus markers

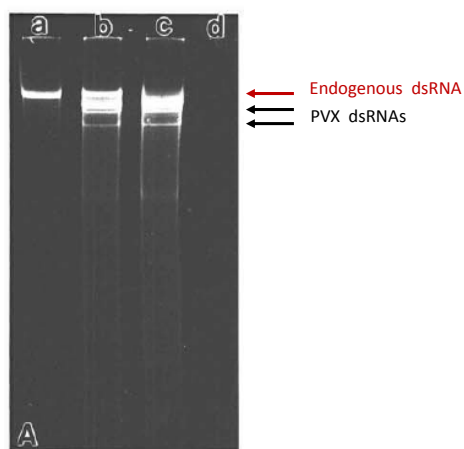


From: Morris, Dodds, Hillman, Jordan, Lommel, and Tamaki (1983) Plant Molecular Biology Reporter 1:27-30.

dsRNA analysis - 12

Example of dsRNAs from pepper (*Capsicum annum* 'Yolo Wonder') and tobacco (*Nicotiana tabacum* 'Turkish') without or with Potato virus X (PVX) infection.

- a. Healthy 'Yolo Wonder' pepper
- b. PVX-infected 'Yolo Wonder' pepper
- c. PVX-infected 'Turkish' tobacco



** Testing appropriate “healthy” controls very important **

From: Valverde, Nameth, and Jordan (1990) Plant Disease 64:255-258 (Special Topic).

dsRNA analysis - 13

Presence of large dsRNAs in “healthy” plants? New genus of dsRNA viruses!

- Endornaviruses (genus Endornavirus) are dsRNA viruses (linear genome 14 kb to 18 kb) that infect plants, fungi, and oomycetes which have recently been given family status (Endornaviridae).
- The plant infecting endornaviruses:
 - do not form virions (no capsid),
 - are present in every tissue,
 - lack cell-to-cell movement,
 - have no obvious effect on the phenotype of the host plant (except for one associated with male sterility in broad bean), and
 - are transmissible only vertically at a very high rate.
 - The putative polyprotein encoded by the single ORF contains a conserved RNA-dependent RNA polymerase domain (RdRp) and other domains that are not conserved among the members.
 - There are 13 complete endornavirus sequences corresponding to eleven recognized or putative species available: six that infect plants (avocado, pepper, rice, broad bean, *Phaseolus vulgaris*), four that infect fungi, and one that infects an oomycete.

From: 9th Report of ICTV, Virus Taxonomy (2011); and Virus Taxonomy July 2014 Release [at <http://www.ictvonline.org> .

dsRNA analysis - 14

The practical disadvantages to the diagnostician of dsRNA analysis include:

1. Not applicable to DNA viruses.
2. For best results, the technique generally does require the use of organic solvents.
3. Some viruses require more than 10.0g tissue for optimum results [e.g., Potyviruses].
4. Several low-titered genera produce extremely low amounts of viral dsRNAs. [Luteoviruses, some Ilarviruses].
5. Some hosts contain mucilaginous compounds, making for v.e.r.y. s.l.o.w. column chromatography.
6. Laborious - requires hours of “hands-on time”.

dsRNA analysis - 15

The practical advantages to the diagnostician of dsRNA analysis include:

1. No knowledge of the possible identity of the virus is required. A single procedure is followed regardless of the source (plant or fungus culture). No antibodies, primers, or probes required!
2. Field samples can be analyzed the day they arrive in the lab. Small (0.5-5.0g) samples are frequently sufficient.
3. The techniques do not require the use of expensive equipment and can be accommodated on a small amount of bench space in most laboratories.
4. The number, size, and intensity of dsRNA segments can be determined by gel electrophoresis within 48 hours of receipt of a sample. No adjustment other than that of scale is required for single or multiple samples.
5. The gel technique is one of the best available for detecting mixed infections, including satellites (and viroids).
6. Virus-like infections will be detected regardless of whether the virus makes functional, or any, virions.
7. The purified dsRNA can be a source of reagents (labeled probes) for detection methods based on hybridization (dot spots, macro- and macroarrays), with or without cloning the nucleotide sequences in the dsRNA; or, as target for sequencing w/o cloning (NGS).
8. The purified dsRNA, when melted, can be a source of inhibitor-free inoculum.

dsRNA analysis

Questions?

Thanks!

...Then lunch break...