Methods in Molecular Biology

Chapter 5 Meta-genome sequencing from mycorrhizal fungi

Title: Detection and characterization of mycoviruses in arbuscular mycorrhizal fungi by deep-sequencing

Running head: Characterization of mycoviruses in AM fungi by deep-sequencing

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Abstract

Fungal viruses (mycoviruses) often have a significant impact not only on phenotypic expression of the host fungus, but also on higher order biological interactions, e.g., conferring plant stress tolerance via an endophytic host fungus. Arbuscular mycorrhizal (AM) fungi in the phylum Glomeromycota associate with most land plants and supply mineral nutrients to the host plants. So far, little information about mycoviruses has been obtained in the fungi due to their obligate biotrophic nature. Here we provide a technical breakthrough, 'two-step strategy' in combination with deep-sequencing, for virological study in AM fungi; dsRNA is first extracted and sequenced using material obtained from highly productive open pot culture, and then the presence of viruses is verified using pure material produced in the *in vitro* monoxenic culture. This approach enabled us to demonstrate the presence of several viruses for the first time from a glomeromycotan fungus.

Key words: Arbuscular mycorrhizal fungi, Deep-sequencing, dsRNA, Mycoviruses, Obligate biotroph

1. Introduction

Fungal viruses (mycoviruses) have been found in a variety of fungi, including the

ascomycetes, basidiomycetes, and deuteromycetes. Their genomes are composed of double-stranded RNA (dsRNA) in most cases and unexceptionally possess an RNA-dependent RNA polymerase (RdRp) gene in the genomes (1). Infections of mycoviruses are asymptomatic in many cases, but often have a significant impact not only on phenotypic expression of the host fungus, but also on higher order biological interactions, e.g., attenuation of virulence of a plant pathogenic fungus (2) and conferring plant thermal tolerance via an endophytic host fungus (3).

Arbuscular mycorrhizal (AM) fungi that belong to the phylum Glomeromycota associate with most land plants and supply mineral nutrients, in particular phosphorus, to the host plants through extensive hyphal networks constructed in the soil (4). The AM association with plant roots occurred more than 400 million years ago, and the coincidence of the appearances of early land plants and AM associations suggests that the plant-AM fungal symbiosis were instrumental in the colonization toward land for primitive plants (5). Although distribution of mycoviruses in AM fungi and their impacts on the plant-fungal interactions are of interest, little information has been obtained due to their obligate biotrophic nature.

One breakthrough for virological study in AM fungi is the establishment of a highly productive open pot culture system for AM fungal mycelia (6, 7). In this system about 1 g

of mycelia could be obtained form 70 – 80 plants grown in growth chambers, which is insufficient for purification of viral particles, but may be sufficient for detection and amplification of viral genomes for sequencing. Purity of fungal material, however, is best in the *in vitro* monoxenic culture (8), although fungal growth is generally poorer than in open culture. To overcome these problems, the 'two-step strategy' in combination with the next-generation sequencing technology has been developed recently (9); dsRNA is first extracted from fungal material produced in the open pot culture and subjected to deep-sequencing, and the presence of viral genomes is subsequently verified by RT-PCR using dsRNA extracted from material produced in the *in vitro* monoxenic culture. This approach enabled us to demonstrate for the first time that glomeromycotan fungi harbor diverse mycoviruses and, further, that a new class of virus found in an AM fungus has a significant impact on spore productivity of the host fungus (9).

2. Materials

2.1. Mass Production of Fungal Material in Open Pot Culture

1. Fungal isolates established from either single or multiple spores (see Note 1).
2. Plant host. Small plants that grow well under low-light conditions (e.g., fluorescent light) are suitable. Dwarf marigold (*Tagetes patula*) and *Lotus japonicus* are ideal plants for mass production of hyphal material in a growth chamber (see Note 2)

3. Washed river sand, autoclaved. A mixture of fine (< 1mm in diam) and coarse (≥ 1 mm) particles at ratios between 30:70 to 70:30 (v:v), preferably 50:50, is required (see Note 3).
4. Plastic pots with drainage holes (6 cm in diam, 120 mL in vol)
5. Cone-shaped nylon-mesh bags for separation of hyphal and root compartments (Fig. 1). Pore-size of the nylon mesh is 37 μm that is small enough to prevent roots from passing, but large enough to allow AM fungal hyphae to pass through. Cut a mesh sheet in a semicircular shape (7 cm in radius for 6-cm pot), fold in the middle, and seal the margin by a heat-sealer (about 26 mL in vol).
6. Low-P nutrient solution: 4 mM NH_4NO_3, 1 mM K_2SO_4, 75 μM $MgSO_4$, 2 mM $CaCl_2$, 50 μM Fe-EDTA, and 50 μM KH_2PO_4. The working solution may be made from concentrated (× 100 – 1,000) stock solutions for each salt. Microelements may not be necessary in sand culture.

2.2. In Vitro Monoxenic Culture

1. Minimal medium (8) solidified with 0.3% gelangum (w/v) in Petri dishes.
2. Ri T-DNA transformed root organ culture. Carrot or chicory hairy roots have widely been used (see Note 4).
3. Chloramine-T solution: 20 g L^{-1} chloramine-T.
4. Antibiotic solution: 0.2 g L^{-1} streptomycin and 0.1 g L^{-1} gentamycin.

5. Melting solution: 0.5 M EDTA

2.3. dsRNA Extraction and Electrophoresis

1. Multi-beads shocker (vibration grinder)
2. 3-mL O-ring sealed plastic tubes fitted to the grinder, liquid nitrogen-resistant.
3. Metal cone fitted to the tube.
4. Extraction buffer: 100 mM Tris-HCl at pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) (w/v), and 0.1% 2-mercaptoethanol (v/v).
5. TE (100 mM Tris-HCl at pH 8.0, 2 mM EDTA)
6. PCI (TE-saturated phenol: chloroform: isoamyl alcohol =25: 24: 1).
7. CIA (chloroform: isoamyl alcohol =24: 1)
8. Absolute ethanol.
9. 3 M sodium acetate (pH 5.2) for ethanol precipitation.
10. DNase I (RNase-free) and S1 nuclease.
11. Autoclaved deionized water.
12. 0.8% agarose gel for electrophoresis
13. SYBER safe DNA gel stain for dsRNA staining
14. 1.5-mL tube (for extraction from spores)
15. Small plastic pestle fitted to 1.5-mL tube (for extraction from spores)
16. Dissecting microscope (for extraction from spores)

2.4. cDNA Amplification and Deep-sequencing

1. Dialysis tube, 25 kDa cut-off
2. First strand cDNA synthesis kit
3. Primers for cDNA amplification (3): 6N-anchor primer, CCTGAATTCGGATCCTCC-NNNNNN; anchor primer, CCTGAATTCGGATCCTCC.
4. *Taq* polymerase
5. Thermal cycler
6. DNA cleanup spin columns
7. Next-generation sequencer (see Note 5).

2.5. Rapid Amplification of cDNA End (RACE)

1. 5'/3' RACE kit, including appropriate primers for cDNA synthesis and amplification.
2. 1st, 2nd, and 3rd specific primers designed based on the 5'- and 3'-end sequences of target dsRNA
3. TA-cloning vector
4. Ligase and buffer
5. *E. coli* JM109 competent cell

6. Medium for blue-white selection: LB medium with 50 mg L^{-1} ampicillin solidified by 1.5% agar to which 20 µL of 100 µM isopropylthio-β-D-galactoside and 35 µL of 50 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-garactoside are applied before use.
7. Sequence primers: U-19mer primer (GTTTTCCCAGTCACGACGT) and T7 promoter primer (CTAATACGACTCACTATAGGG).
8. Dideoxy-cycle sequencing kit.
9. Capillary sequencer.

2.6. Sequence Analysis and Phylogeny

1. BLAST program (http://www.ncbi.nlm.nih.gov/).
2. Pfam database (http://pfam.sanger.ac.uk/).
3. MEGA 5 software for alignment and phylogeny (http://www.megasoftware.net/) (10).

2.7. Detection of Mycoviruses from In Vitro culture

1. A primer for first strand cDNA synthesis (virus-specific or 6N random primer).
2. Virus-specific primer pairs for the 1st and 2nd (nested) PCR.

2.8. Establishment of Single Spore-Culture Lines for Virus Elimination

1. *Lotus japonicus* or marigold as host plant.

2. Washed river sand, autoclaved.
3. Small (6 cm in diam, 120 mL in vol) and medium (9 cm in diam, 350 mL in vol) plastic pots with a drainage hole
4. Low-P nutrient solution.
5. Dissecting microscope

3. Methods

A key step for successful detection of dsRNA in AM fungi is mass production of fungal material; at least 0.5 g of fresh mycelia is necessary for detection of major dsRNA segments in electrophoresis. Representative results are shown in Fig. 2. Deep-sequencing is much more powerful than electrophoresis for detection of dsRNA, and, in fact, we unexpectedly found a viral segment from a fungus in which no dsRNA was observed in electrophoresis during transcriptome analysis by the Illumina RNA-seq (Kikuchi and Ezawa, unpublished observation). It is also true, however, that deep-sequencing is still costly at present, and thus one might not apply the method unless dsRNA segments are detected in electrophoresis. Therefore, electrophoresis is the first key analysis in the virological study of the fungi.

Among factors that influence productivity of mycelium, fungal species is most critical; some species are highly productive in sand culture, but some are not. Water regimes also

critically affect mycelial yield. Tips for optimizing the growth conditions of the fungi are suggested in this section. After successful detection and sequencing of viral segments, it is highly recommended to validate their presence in the fungus grown *in vitro* as long as the segments are detected in the material obtained from open culture. RT-(nested) PCR using viral sequence-specific primers is sensitive enough to detect viral segments from a small amount of fungal material.

3.1. Mass Production of Fungal Material in Open Pot Culture

1. About 100 mL sand is put into the pots, watered lightly, and the surface is shaped to fit to the cone-shaped mesh bag. After fitting the mesh bag, the bag is filled with sand (see Note 6). Plant seeds are sowed in the middle of the bag, inoculated with AM fungal spores (e.g., 500 – 1,000 spores pot^{-1} for *Rhizophagus* spp.), and grown for 6 – 8 weeks (see Note 7) in a growth chamber at a photosynthetic photon flux of 150 μmol m^{-2} s^{-1} (20 – 25˚C; 16-h day length; 60 – 70% relative humidity).
2. A sufficient amount of the low-P nutrient solution is given until the solution flowed out from the drainage at appropriate intervals (see Note 8). Accumulated salts in the medium may be washed out with deionized water once a week.
3. At harvest, the mycorrhizal compartment in which the plants are growing is removed together with the mesh bag, and mycelia in the hyphal compartment are collected on a stainless steel mesh (50 μm opening) by wet sieving as quickly as

possible, blotted on a paper towel, transferred into a plastic tube, frozen in liquid nitrogen, and stored at -80˚C. Three to five pots could be processed together, which will save time and also improve recovery of mycelia. In the case of *Rhizophagus clarus* strain RF1 (= *Glomus* sp. strain RF1), 10 – 30 mg FW mycelium pot^{-1} could be obtained under the conditions.

3.2. In Vitro Monoxenic Culture

1. Spores are surface sterilized with the chrolamine-T solution for 10 min, followed by the antibiotic solution for 10 min, and then washed with deionized water.
2. Ri T-DNA transformed roots maintained on the Minimal medium are inoculated at five spores $plate^{-1}$ and incubated in the dark at 27˚C for 10 weeks.
3. Spores produced in the medium are collected on a 53 μm stainless mesh after melting the gel by shaking in the melting solution and subjected to dsRNA extraction.

3.3. dsRNA Extraction and Electrophoresis

1. The frozen mycelia (0.5 – 1.0 g) are ground in the presence of liquid nitrogen in the 3-mL tube with the metal cone using the Multi-beads shocker at 3 × 15 s at 2,000 rpm, suspended in 4 ml of the extraction buffer, extracted twice with an equal volume of PCI and once with CIA, precipitated with 2 volumes of ethanol and 1/10

volume of 3 M sodium acetate, and then dissolved in 50 – 100 μL of TE.

2. In the case of dsRNA extraction from spores, approx. 200 spores are crushed in 100 μL of the extraction buffer in a 1.5-mL tube with the small pestle under a dissecting microscope and then the slurry was subjected to PCI/CAI extractions.
3. To digest genomic DNA and single-stranded RNA, the fraction is incubated twice with 0.4 units μL^{-1} DNase I and 3.4 units μL^{-1} S1 nuclease at 37ºC overnight, precipitated by ethanol after PCI/CAI extractions, dissolved in 25 μL deionized water.
4. The dsRNA fraction is subject to electrophoresis and visualized with the SYBER safe.

3.4. cDNA Amplification and Deep-sequencing

1. dsRNA segments are eluted from the gel pieces in the dialysis tube, digested again by overnight incubation with DNase I and S1 nuclease, extracted with PCI/CAI, precipitated with ethanol, and then dissolved in 25 μL deionized water.
2. First strand cDNA is synthesized with 2 ng dsRNA and 800 ng 6N-anchored primer in a final volume of 20 μL according to the instruction of the kit.
3. The cDNA is randomly amplified with *Taq* polymerase using 1 μL cDNA solution and 5 μM anchor primer in a final volume of 15 μL with the following thermal cycle program: initial denaturation at 94˚C for 2 min, followed by 40 cycles of 94˚C

for 15 s, 55˚C for 30 s and 72˚C for 2 min, and final extension at 72˚C for 10 min. The PCR product is purified by the DNA cleanup spin column.

4. The amplified cDNA is sequenced with a next-generation sequencer and assembled with appropriate software (see Note 9).

3.5. RACE

1. The extreme end of the target dsRNA is reverse-transcribed with the 1st specific primer at 55 – 60˚C and poly(A)-tailed according to the instruction of the RACE kit.
2. The poly(A)-tailed cDNA is amplified with *Taq* polymerase using the oligo(dT)-anchor primer in combination with the 2nd specific primer with the following program: initial denaturation at 94˚C for 2 min, 30 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 1 min and final extension at 72˚C for 10 min.
3. The first PCR product is further amplified with the anchor and the 3rd specific primers with the same thermal cycle program.
4. The second PCR product (0.2 – 0.4 pmol) is ligated to the vector at 16˚C for 40 min, and *E. coli* competent cell is transformed by heat shock at 42˚C for 45 s. The transformants are incubated on the blue-white selection medium at 37˚C over night, and the insert-positive (white) clones are selected.
5. The target insert is amplified by colony PCR using U-19mer and T7 promoter

primers with the following program: initial denaturation at 94˚C for 2 min, 30 cycles of 94˚C for 30 s, 50˚C for 30 s and 72˚C for 1 min and final extension at 72˚C for 10 min, electrophoresed on a 1.0 – 1.2% agarose gel, and visualized with SYBER safe.

6. The amplicon is extracted from the gel and purified with the spin column. Sequence reaction is performed using 10 – 20 ng of the purified amplicon according to the instruction of the sequence kit, and the product is sequenced with a capillary sequencer.

3.6. Sequence Analysis and Phylogeny

1. Large (> 1 kbp) contigs are subjected to BLASTn search, and those show similarities to viral sequences are analyzed further.
2. Motif searches are performed by Position-Specific Iterated BLAST (PSI BLAST) against Pfam database. Multiple sequence alignments and phylogenetic analysis with the neighbor-joining or maximum-likelihood method are implemented by MEGA5.

3.7. Detection of Mycoviruses from In Vitro culture

1. First strand cDNA is synthesized using dsRNA prepared from the *in vitro*-produced spores using the virus-specific or 6N-random primer.
2. The cDNA is amplified by nested PCR using the 1st and 2nd PCR primers with

appropriate thermal cycle programs.

3. The final PCR product is purified, cloned, and sequenced by a capillary sequencer.

3.8. Establishment of Single Spore-Culture Lines for Virus Elimination

1. Washed river sand is put into a 6-cm pot, and seedlings of *L. japonicus* (two plants pot^{-1}) are grown for a few days in the growth chamber.
2. Spores obtained either by open pot culture or by *in vitro* culture were collected under a dissecting microscope.
3. A small pit (1 – 2 cm depth) is made in the vicinity of the roots with a plastic tip that fits to a 200-µL pipet ('yellow tip'), and a single spore is put and covered with sand.
4. The seedlings are grown for 6 – 7 weeks with the low-P nutrient solution in a growth chamber (first multiplication).
5. The *L. japonicus* seedlings are non-destructively transferred to a 9-cm pot with the medium (see Note 10), and then the pot is filled with sand. Marigold is sown around the seedlings (2 – 3 seeds pot^{-1}) and further grown with the low-P nutrient solution for 8 – 10 weeks in a greenhouse (second multiplication). Spores are harvested by wet sieving (see Note 11). The presence or absence of viral segments is examined by RT-PCR using a dsRNA fraction prepared from the spores.

4. Notes

1. Viruses may occasionally be lost during single spore isolation.
2. C_3 plants may be more suitable for fungal propagation in a growth chamber i.e. under low-light conditions. Generally productivity of C_4 plants is greater than that of C_3 plants in a greenhouse (with sunlight), but not in a growth chamber.
3. Coarse sand provides aerobic conditions that are essential for hyphal growth of AM fungi.
4. The AM fungus-free root organ culture could be purchased from Glomeromycota In vitro Collection (http://www.agr.gc.ca/eng/?id=1236786816381).
5. Many companies provide sequencing and subsequent assembling service using next-generation sequencers such as Roche 454 GS Titanium and Illumina HiSeq.
6. For species that do not fit to sand culture and require a certain soil for successful colonization, a (autoclaved) soil-sand mixture at a ratio of 1:9 (v:v) may be used in the mycorrhizal compartment (within the mesh bag). The medium in the hyphal compartment from which mycelia are collected, however, should be 100% sand for maximum RNA yield.
7. Appropriate culturing period will vary among fungal species. Some species grow rapidly, but senesce rapidly (e.g., *Rhizophagus irregularis*), for which a shorter culturing period may result in higher yield of active mycelia. Whereas some species

senesce slowly (e.g., *Rhizophagus clarus* and *Acaulospora colombiana*), for which maximum yield of active mycelia may be expected 7 – 8 weeks after sowing.

8. The plants should be grown with minimum water (nutrient solution), which is essential to maximize mycelial yield. Too much water creates anaerobic conditions under which growth of the aerobic fungi is suboptimal.
9. The scale of sequencing depends on number of viruses in the fractions, their genome size, and amount of contaminant RNA, e.g., rRNA. Prior to deep-sequencing, we strongly recommend to construct a clone library of the randomly amplified cDNA for sequencing of at least 10 – 20 clones by a conventional sequencer, from which frequency of viral cDNA can be estimated.
10. The plants and medium should carefully be taken out from the pot in a non-destructive manner before transplanting. Fungal biomass is usually very small at the end of first multiplication, and thus the second multiplication is essential before examining spore production.
11. Single spore culture may usually be established from 20 – 50% of the pots in the case of *Rhizophagus* spp.

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Figures

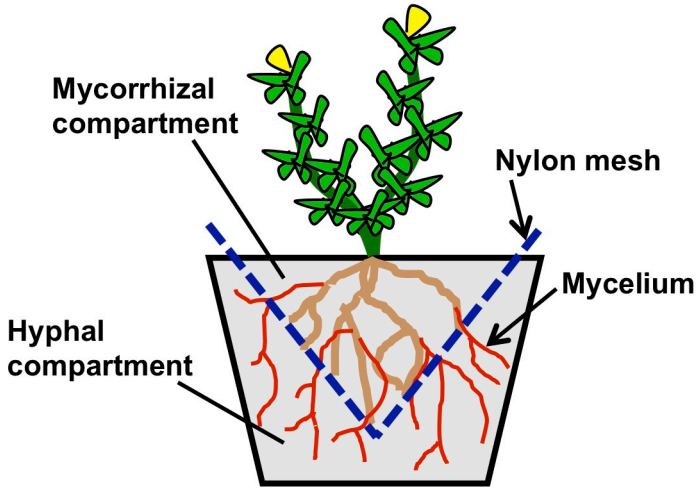

Fig. 1. Mesh bag-separated open pot culture system. The dotted line represents the 37-µm nylon mesh bag that separates the mycorrhizal and hyphal compartments. The pore size of the mesh is small enough to prevent roots from passing through, but large enough to allow passage of AM fungal hyphae.

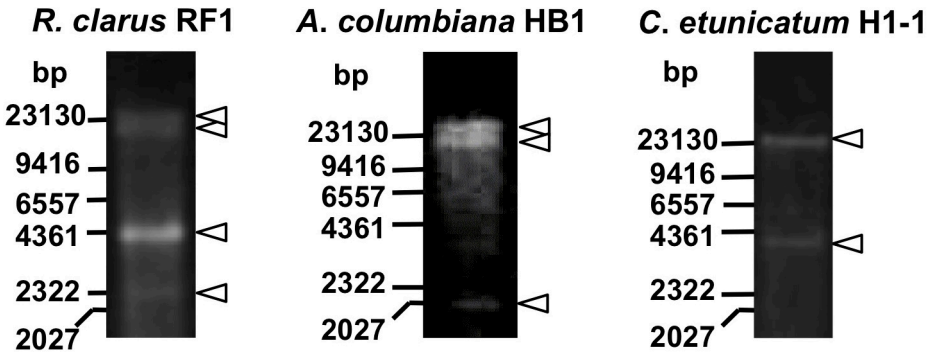

Fig. 2. Electrophoretic analysis of dsRNA fractions prepared from extraradical mycelia of *Rhizophagus clarus* strain RF1, *Acaulospora colombiana* strain HB1, and *Claroideoglomus etunicatum* strain H1-1. Total nucleic acid was extracted from 0.5 – 1.0 g mycelia by the SDS-phenol method, digested by DNase I and S1 nuclease, and subjected to electrophoresis. Arrowheads indicate dsRNA segments.