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ORIGINAL ARTICLE

Deep sequencing evidence from single grapevine plants reveals a virome dominated by mycoviruses

M. Al Rwahnih · S. Daubert · J. R. Úrbez-Torres · F. Cordero · A. Rowhani

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Abstract We have characterized the virome in single grapevines by 454 high-throughput sequencing of double-stranded RNA recovered from the vine stem. The analysis revealed a substantial set of sequences similar to those of fungal viruses. Twenty-six putative fungal virus groups were identified from a single plant source. These represented half of all known mycoviral families including the *Chrysoviridae, Hypoviridae, Narnaviridae*, *Partitiviridae,* and *Totiviridae*. Three of the mycoviruses were associated with *Botrytis cinerea*, a common fungal pathogen of grapes. Most of the rest appeared to be undescribed. The presence of viral sequences identified by BLAST analysis was confirmed by sequencing PCR products generated from the starting material using primers designed from the genomic sequences of putative mycoviruses. To further characterize these sequences as fungal viruses, fungi from the grapevine tissue were cultured and screened with the same PCR probes. Five of the mycoviruses identified in the total grapevine extract were identified again in extracts of the fungal cultures.

Introduction

Grapevine hosts the widest variety of pathogens of any woody agricultural plant [1]. Virus infection is of particular concern in this host; at least 60 different viruses have been found to infect grapevine. The fungi, including their pathogenic members, are also known to be well represented in grapevine; single vines may support scores of fungal species [2].

For a census of the viruses in grapevine, including viruses that infect the fungi found on grapevine, the metagenomic strategy of whole-community sequencing [3] may be the most effective method. The fungal hosts of mycoviruses may occur at very low titers on their plant hosts, and they can be difficult to culture. Direct recovery of mycoviral particles is impractical for mycoviruses at low titers or for unencapsidated mycoviral forms. Compared with other methods of census, whole community genomic analysis is less disadvantaged by these conditions and may allow for the characterization of a fungal virome that would otherwise remained largely undescribed. This approach has previously been used to characterize plant virus infection in grapevine [4, 5].

Mycoviruses show no extracellular phase to their infection cycles; they are not seen to move cell to cell [6]. The genomes of most mycoviruses consist of dsRNA [7]. Here, we have subjected dsRNA preparations from grapevine (*Vitis vinifera)* to analysis by high-throughput sequencing and have made a census of the putative mycoviruses that occur in aerial stems of this host. This approach has revealed that, in the example presented here, fungal virus-like sequences represented the most prevalent and diverse viral group in the vine stem environment.

Materials and methods

Sample preparation

Two grapevines from a U. C. Davis collection, sister clones Syrah B0 and Syrah B1, maintained in a lath house

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M. Al Rwahnih · S. Daubert · J. R. Úrbez-Torres · F. Cordero · A. Rowhani (✉)
Department of Plant Pathology, University of California, Davis, California, 1 Shields Avenue, Davis, CA 95616, USA
e-mail: akrowhani@ucdavis.edu

environment, were used in this study. In each case, dsRNA was extracted from 90 g of bark scrapings as described [8], but without the enzymatic digestion step using DNase and RNase. Ten-microliter aliquots of the dsRNA were analyzed by electrophoresis in 1% agarose gels in TAE buffer, against 1K plus DNA size standards (Invitrogen). Complementary DNA (cDNA) libraries were synthesized using a SuperScript® II Reverse Transcriptase (RT) Kit (Invitrogen) primed with random hexmers (300 ng/µl, Invitrogen) and amplified using a GenomePlex® complete whole-genome amplification kit (Sigma, San Louis, MO). The amplified DNA preparation was cleaned, and DNA quality was checked as described before [4]. Samples were subjected to 454 Life Sciences (Branford, CT, USA) high-throughput pyrosequencing, using the Genome Sequencer FLX platform. Additional grapevine control samples from the collections at U. C. Davis that were used here were Syrah 99, Syrah 525, Syrah 877, Pinot Noir 23, Pinot Noir 2A and Chardonnay 4.

Bioinformatic analysis

The High-Speed Sequence Search Suite (HS^3) algorithm from GenomeQuest (Westborough, Mass.) was used as described before [4]. Reads were assembled into larger contigs using 454 Newbler Assembler software (454 Life Sciences, Branford, CT, USA). Contigs were subjected to both BLASTN and BLASTX analysis [9] using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov).

PCR analysis

Primers designed from contig sequences were used in PCR analysis of the cDNA libraries using the GoTaq kit from Promega (Madison, WI, USA). The PCR mixture contained 5 µl 5xGO Taq PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl each of 10 mM primers, 0.5 units of Taq DNA polymerase (Promega, Madison WI, USA) and sterile water to a final volume of 24 µl; 1 µl cDNA was added directly to tubes. The denaturation step was at 93°C for 4 min; there were 35 cycles of amplification (94°C for 30 sec, 57°C for 45 sec and 72°C for 1 min), with a final extension for 7 min at 72°C. ssRNA was prepared as above from uninfected Thompson Seedless grape material from tissue culture, for use as a healthy control. PCR products were analyzed by electrophoresis in 1.2% agarose gels in TAE buffer, compared with 1 Kb Plus DNA Ladder (Invitrogen) size standards, stained with ethidium bromide. PCR products were eluted from agarose gels using a ZymoClean Gel DNA Recovery Kit (Zymo-Research, Orange, CA) and submitted for direct sequencing at the University of California, Davis sequencing facility. For RT-PCR analysis, the original dsRNA preparation was denatured at 95 °C for 5 min and placed on ice, and one µl was then used in RT-PCR as described in ref. 4 with an annealing temperature of 57 °C.

Fungal culture

Fungi were isolated from several lignified grapevine shoots of both Syrah-B0 and Syrah-B1 vines as described in ref. [10]. Shoots were cleaned of loose bark and surface-disinfected by treatment with 0.5% sodium hypochlorite for 5 min. After air-drying, the surface tissue was cut away to expose the vascular wood. Pieces of wood tissue (9 mm^2) were placed on 90-mm Petri dishes (Fisher Scientific, Santa Clara, CA) containing 4% potato dextrose agar (DIFCO™, Detroit, MI) amended with 100 ppm tetracycline hydrochloride (Sigma-Aldrich, St Louis, MO). Cultures were incubated at room temperature until fungal colonies were observed. Fungal colonies were isolated by excising hyphal tips from colony margins onto fresh plates. Fungal species isolated from grapevine were initially identified by colony and spore morphology.

Extraction of nucleic acids from fungal culture

For virus detection we used miniscale dsRNA extraction as described [11]. Syrah B0 and B1 extracts were used as positive controls for PCR analysis, sequencing and BLAST analysis as described above. For identification of fungal species by internal transcribed spacer (ITS) analysis, DNA was extracted from cultured mycelia using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) as recommended by the manufacturer. Oligonucleotide primers ITS1 and ITS4 were used to amplify the internal transcribed spacer region of the nuclear ribosomal DNA, including the 5.8S gene, as described before [12]. The ITS region was sequenced in both directions, and BLAST analysis was done as described above.

Results

Grapevine canes from samples SyB0 and SyB1 showed no outward signs of fungal infection, but dsRNA samples from parallel extractions of that material showed a series of strongly staining bands after gel electrophoresis, with mobilities corresponding to lengths of 2-3 kbp (Fig. S1). Reverse transcription of the two dsRNA samples produced cDNA libraries that were analyzed by 454 Life Sciences (Branford, CT, USA) high-throughput pyrosequencing with the Genome Sequencer FLX as described before [4]. The analysis generated 34 megabases of sequence data (Table S2). The sequence data derived from each of the two plants that

were extracted in parallel were found to be very similar, and the two datasets were pooled for the further analysis presented in this report. From a total of 156,421 high-quality reads, comparative analysis [9] against the GenBank database showed that 20,614 of the fragments were associated with fungi.

Characterization of the viral families present in grapevine

A total of 40,974 fragments were identified as viral in origin. Of those fragments, 19,970 were identified as belonging to grapevine rupestris stem pitting-associated virus (GRSPaV; genus *Foveavirus*, family *Betaflexiviridae*), the only infectious plant virus identified in this study.

A total of 21,004 fragments were identified as mycovirus-like sequences. They were grouped by their homology with GenBank sequences from members of seven mycovirus families, plus another group not assigned to family (Table 1). The numbers of mycovirus-like fragments for members of these families were all lower than the number of fragments associated with the coincident GRSPaV infection.

Contigs were built from those viral fragments that shared 92 percent or greater nucleotide sequence similarity with each other, over a minimum of 90 nucleotides of overlap. Two hundred eight contigs were assembled (Table 1).

Identification of mycoviruses

BLASTN analysis showed that five groups of these contigs corresponded to sequences with known mycoviruses (Table 1). None of the other contigs had significant nucleotide sequence homology with sequences from the GenBank database associated with any known virus. BLASTN alignment data are given in Appendix 1 (Electronic Supplementary Material).

Identification of putative mycoviruses at the familial level

Using BLASTX analysis, contigs were categorized by their similarity with GenBank reference protein sequences. Categories were identified with 25 known mycoviruses and one cryptovirus (Table 1). Those sequences from the GenBank database are listed in Table 1 as "reference sequences."

The percentage BLASTX identity of each contig (Table 1) with its reference protein sequence, calculated using the default BLASTX criteria, showed a range that varied from greater than 90 percent to less than 30 percent. With the exception of a subset of the contigs similar to viruses infecting *Botrytis* (Botryotinia fuckeliana totivirus 1; Botrytis virus F), most of the contigs were less that 80 percent similar at the protein sequence level to their GenBank viral reference sequences. The median similarity in Table 1 was 51 percent. BLASTX alignment data are given in Appendix 2 (Electronic Supplementary Material).

The most prevalent generic categories were populated by contigs having sequences similar to viruses from the fungal hosts *Penicillium chrysogenum*, *Fusarium graminearum*, *Botrytis cinerea*, and *Cryphonectria parasitica*. The sequences of all viral contigs referenced by number in this report were deposited in GenBank, with accession numbers GU108589 to GU108601 (Table S6).

Direct viral detection

An attempt was made to detect the presence of some of these putative mycoviruses by direct PCR amplification of their genomic sequences from the cDNA library transcribed from the original dsRNA preparations. Specific PCR primers (Table S3) were designed for the detection of sequences from fifteen viral contigs, among which the five most prevalent mycovirus families from Table 1 were represented. Products of the PCR reactions were analyzed on 1.5% agarose TAE gels. In all cases, products of the predicted sizes were produced (Table 2; examples of the gel analysis are shown in Fig S4a). Parallel analysis, using RT-PCR, with the original dsRNA prep as template, gave similar positive results (Fig. S4b). The mycoviral sequences for each of these PCR products, as predicted by the BLAST identities listed in Table 1, were confirmed by direct sequencing of each (their accession numbers are in Table 2). The putative viruses from which these PCR products were derived have been given provisional names (Table 2) reflecting their grapevine origins and their families.

Identification of mycoviruses in fungi cultured from grapevine

In a further effort to identify these sequences as mycoviral, fungi were cultured directly from the vine stem material. Eleven fungal species from cultures growing on synthetic medium were identified (Table 3) by their ITS sequences. The accession numbers of the sequences of those PCR products primed by ITS-1- and ITS-4-specific primers on nucleic acid preparations extracted from the fungal cultures are given in Table 3.

We tested those cultures for viruses, using the same PCR primers (Table S3) that we used for virus identification from the initial grapevine extracts. Five of the mycoviruses identified in Table 1 were identified again (Table 4), each from one or two of the fungal cultures. These viruses had sequences most closely related to

Table 1 Category list of putative grapevine associated mycovirus

Family	Reference sequence	Blastn identity %				Blastx identity %							
		No. of fragments	No. of contigs	70–80	80–90	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
Chrysoviridae	Penicillium chrysogenum virus	3185	44			4	21	6	7	6			
	Cryphonectria nitschkei chrysovirus 1	148	10			4	1	3	2				
	Helminthosporium victoriae 145S virus	84	1				1						
Totiviridae	Botryotinia fuckeliana totivirus 1	2172	22	4	5		1	1			1	12	7
	Saccharomyces cerevisiae virus L-A (L1)	14	3				2	1					
	Amasya cherry disease-associated mycovirus	804	2			1		1					
	Ophiostoma minus totivirus	212	1							1			
	Helminthosporium victoriae virus 190S	216	1						1				
	Black raspberry virus F	420	5			2	3						
	Sphaeropsis sapinea RNA virus 1	93	1				1						
	Magnaporthe oryzae virus 1	118	1			1							
Partitiviridae	Gremmeniella abietina RNA virus	255	9		1	2	3	3				1	
	Fusarium poae virus 1	1311	4		1				1	2	1		
	Aspergillus ochraceous virus	380	1							1			
	Mycovirus FusoV	188	1							1			
	Pepper cryptic virus 1	49	1				1						
Narnaviridae	Ophiostoma mitovirus 4	347	6					2	3	1			
	Cryphonectria parasitica mitovirus 1-NB631	18	1						1				
	Thielaviopsis basicola mitovirus	27	1						1				
	Botrytis cinerea debilitation-related virus s	10	1					1					
Hypoviridae	Cryphonectria hypovirus 3	1088	15		2				7	4	3	1	
Gammaflexiviridae	Botrytis virus F	39	5		4			1				2	2
Alphaflexiviridae	Botrytis virus x	26	1			1							
Unassigned viruses	Fusarium graminearum dsRNA mycovirus-1	7744	58			5	18	13	8	8	4	2	
	Diaporthe ambigua RNA virus 1	1497	10				2	5	1	2			
	Curvularia thermal tolerance virus	559	3					1	2				
	Total	21004	208										

Penicillium chrysogenum virus 1, Cryphonectria hypovirus 3, Mycovirus Fuso V, Botrytis virus F and Botrytis virus X. *Penicillium* and *Botrytis* fungal species had been identified in the cultures by their ITS sequences (Table 3), so they were potential hosts for three of the mycoviruses identified from the cultures listed in Table 4.

Table 2 Putative mycoviral sequences amplified from grapevine extract by PCR

Accession number		Contig		"Reference sequence" (blastx)	Blastx	
		Length (nt)	Number		Identity %	Coverage %
GaPV-1	*GU108584*	2231	474	Fusarium poae virus 1	77	89
GaPV-2	*HM852916*	978	30	Mycovirus FusoV	63	89
GaHV-1	*GU108591*	1117	195	Cryphonectria hypovirus 3	71	94
	GU108593	853	486	Cryphonectria hypovirus 3	67	88
	GU108592	907	517	Cryphonectria hypovirus 3	59	90
	HM852914	301	286	Cryphonectria hypovirus 3	82	98
	HM852915	580	556	Cryphonectria hypovirus 3	73	99
GaNV-1	*GU108590*	622	501	Ophiostoma mitovirus 4	50	98
	GU108586	538	157	Ophiostoma mitovirus 4	44	69
GaCV-1	*GU108588*	2879	446	Penicillium chrysogenum virus	61	99
GaCV-2	*GU108589*	945	208	H. victoriae 145 S virus	26	31
GaMV-1	*GU108587*	1225	150	Black Raspberry virus F	36	78
GaTV-1	*GU108585*	4218	412	Amasia Cherry mycovirus	32	59
GaGV-1	*HM852917*	672	278	Botrytis virus F	43	80
GaAV-1	*HM852918*	702	162	Botrytis virus X	27	59

Table 3 Accession numbers of ITS sequences used to identify fungal species cultured from grapevine

Isolate	Species	Blastn		Accession no.
		Query coverage %	Identity %	
UCD1Syrah	*Penicillium* sp.	100%	97%	HM849046
UCD2Syrah	*Botryotinia fuckeliana*	99%	100%	HM849047
UCD3Syrah	*Botryotinia fuckeliana*	100%	99%	HM849048
UCD4Syrah	*Alternaria* sp.	100%	99%	HM849049
UCD5Syrah	*Hypocrea lixii*	100%	99%	HM849050
UCD6Syrah	*Aspergillus* sp.	100%	100%	HM849051
UCD7Syrah	*Cladosporium* sp.	100%	100%	HM849052
UCD8Syrah	*Epicoccum nigrum*	100%	99%	HM849053
UCD9Syrah	*Bionectria* sp.	100%	99%	HM849054
UCD10Syrah	*Pestalotiopsis* sp.	100%	99%	HM849055
UCD11Syrah	*Cladosporium* sp.	100%	99%	HM849056
UCD12Syrah	*Aureobasidium pullulans*	100%	99%	HM849057
UCD13Syrah	*Bionectria* sp.	100%	99%	HM849058

Neither *Cryphonectria* nor *Fusarium* fungal species were identified by ITS analysis of the fungi growing on culture plates from which we identified the viruses closest in sequence to Cryphonectria hypovirus 3 or Mycovirus Fuso V, and *Cryphonectria* or *Fusarium* fungal species were not identified in the initial BLAST analysis of fungal sequences present in the cDNA library generated from grape stem. Specific fungal hosts from grapevine for these Fusarium virus- and Cryphonectria virus-like viruses remain to be identified. Attempts to identify the fungal hosts of each of these five viruses unambiguously by pure culture fungal isolations are continuing.

Discussion

A surprising diversity of genomic sequences tentatively identified as mycoviral is described here. These sequences were observed in the epiphytic fungal habitat on aerial stems from a single selection of grapevine. A metagenomic approach allowed whole-community sampling of that habitat. The virome that was characterization here appears to reflect a range of fungal hosts, but these have been neither cultured well nor characterized. As was also found in metagenomic studies of aquatic or medical habitats [13–16], most of the genomic sequences identified here

Table 4 Mycoviral sequences amplified from fungal culture extract by PCR

Contig		"Reference sequence" (blastx)	Blastx		
Length (nt)	Number		Identity %	Coverage %	Gene name
580	556	Cryphonectria hypovirus 3	73	99	Polyprotein
301	286	Cryphonectria hypovirus 3	82	98	Polyprotein
1117	195	Cryphonectria hypovirus 3	71	94	Polyprotein
2879	446	Penicillium chrysogenum virus	61	99	RdRp
978	30	Mycovirus FusoV	63	89	RdRp
672	278	Botrytis virus F	43	80	Replicase
702	162	Botrytis virus X	27	59	Replicase

appear to represent previously unknown viruses. These findings exemplify the ecological complexity that can be expected in the deep characterization of terrestrial microhabitats, including those in the agricultural realm.

The infection of fungi by mycoviruses is extensive and well documented [6]; fungal viruses may be as widespread and diverse as the viruses that infect plants [7]. Since grapevine is known to support a range of fungal species, we might well have predicted that a comprehensive description of grapevine viruses would include a significant number of mycoviruses.

The cohort of mycoviruses found in this grapevine sample was more extensive than that typically observed in other deep-sequencing analyses of grapevine material (e.g., see Refs. [4, 5]). The enhanced mycovirus titer could have arisen in this case as a consequence of the lathe house growing conditions of the sample (e.g., compare with the extract of the field-grown sample SY-877, Fig. S1).

Evidence for more than 26 different mycovirus-like genomes was found in the sample

Sequences similar to GenBank entries associated with five known mycoviruses were identified in the BLASTN (Table 1) analysis. None of the other viral genomic sequences listed in Table 1 could be associated with known viruses by BLASTN analysis. Three additional sets of viral sequences were identified as mycoviral in origin (Table 4) due to their recovery from fungal cultures (derived from the grapevine source material).

The survey identified 26 categories of sequences at the mycoviral familial level (Table 1) by BLASTX analysis. Within seven of those categories in Table 1 there was a span of 40 percent or more in the percentage variation among contigs versus the GenBank reference sequence. In those cases, multiple different viruses may be present in single categories.

For example, one category of contigs in Table 1 (line 4) contained sequences related to Botryotinia fuckeliana totivirus 1. One of the contigs (number 186) was only 35 percent identical to the GenBank reference sequence (over a span of 281 amino acids). Contig 473 from the same category was 95% identical to the reference sequence. Variation between the inferred translation products of these two contigs is shown in Fig S5a. Variation of 50 percent or more at the protein sequence level may be indicative of separate totiviruses [17], so if the variation rates shown between these contigs were representative of their respective genomes, at least two separate viruses would be represented in this category in Table 1. A similar distant similarity alignment from the Penicillium chrysogenum virus category is shown in Fig. S5b. Thus, the total number of different mycovirus genomes represented in the table would appear to be greater than the 26 categories listed there.

Unidentified viruses related to Fusarium gramminarium mycovirus and to Cryphonectria hypovirus

Most of the viral sequences described here could not be identified by BLASTN analysis, and so they appear to represent previously undescribed viruses. The longest contigs to be assembled here were identified as similar to Fusarium gramminarium mycovirus 1. Contig 341 (6595 nt; originating from Syrah B0 material) and contig 198 (4967 nt; originating from Syrah B1) were assembled independently. They were found to be identical to each other over their region of overlap. BLASTX analysis of that region (with 95 percent coverage) showed only 43 percent identity with the F. gramminarium mycovirus 1 reference sequence from GenBank.

A set of sequences most closely related to Cryphonectria hypovirus 3 was identified in the initial grapevine extract, and again in extracts of fungi cultured from the grapevine source. More than three kbp of the reference genomic sequence from the GenBank database was covered by these contigs (listed in Table 2). The total length of these contigs averaged 84 percent identity (with 67 percent coverage) over the homologous sections of the Cryphonectria hypovirus 3 genome at the nucleotide sequence level.

The fungal host of Cryphonectria hypovirus 3 is the agent of chestnut blight. This fungus has not been reported to be found on grapevine, and no evidence of its presence was found among the fungal hits from the BLAST analysis of the cDNA library initially isolated from grapevine. No evidence of its presence was found by sequencing the specific ITS-1 and -4 PCR products (Table 3) amplified from the fungal cultures in which three of these hypovirus-specific contigs were identified. Thus, the virus represented by the contigs similar to Cryphonectria hypovirus 3 appears to infect a fungal host different from the host of the most similar virus in the GenBank database. Other viruses related to Cryphonectria hypovirus 3 have also been characterized from non-*Cryphonectria* fungal hosts [18, 19].

We also found no evidence for *Fusarium* fungal hosts in this study, despite extensive viral contig sequences similar to sequences from Fusarium gramminarium mycovirus 1. Thus, the two most prominent unidentified mycoviruses here would appear to infect different fungi from the hosts of their most closely related viruses in the GenBank database.

Other unidentified mycovirus-like genomic sequences described here may have been derived from cryptoviruses. Table 1 (line 16) lists the example of a contig distantly related to pepper cryptic partitivirus. Cryptoviruses are mycovirus-like particles that, like mycoviruses, have no extracellular phase to their life cycle and have not been shown to move from cell to cell, but unlike mycoviruses, they are thought to infect plant cells [20].

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