Journal of General Virology (2004), **85**, 3437–3448 DOI 10.1099/vir.0.80293-0

Complete genome sequence of *Mycoreovirus-1*/Cp9B21, a member of a novel genus within the family *Reoviridae*, isolated from the chestnut blight fungus *Cryphonectria parasitica*

Nobuhiro Suzuki,[1] S. Supyani,[1] Kazuyuki Maruyama[1] and Bradley I. Hillman[2]

[1]Agrivirology Laboratory, Research Institute for Bioresources, Okayama University, 2-20-1 Chu-ou, Kurashiki, Okayama 710-0046, Japan

[2]Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901-8520, USA

Correspondence
Nobuhiro Suzuki
nsuzuki@rib.okayama-u.ac.jp

Bradley I. Hillman
hillman@aesop.rutgers.edu

Received 19 May 2004
Accepted 19 July 2004

Mycoreovirus 1 (MYRV-1) is the type species of the newly described genus *Mycoreovirus* of the large virus family *Reoviridae*. The virus was isolated from a hypovirulent strain (9B21) of the chestnut blight fungus, *Cryphonectria parasitica*. A previous study showed that double-shelled particles introduced to fungal spheroplasts resulted in stably infected colonies. Of the 11 double-stranded RNA genomic segments (S1–S11), the three largest (S1–S3) were sequenced previously and shown to have moderate levels of similarity to the homologous segments of mammal-pathogenic coltiviruses (*Eyach virus* and *Colorado tick fever virus*) and another fungus-infecting reovirus, *Mycoreovirus 3* of *Rosellinia necatrix* strain W370 (MYRV-3/RnW370). The sequences of the remaining segments (S4–S11) are reported here. All of the segments have single ORFs on their positive strands and the terminal sequences 5′-GAUCA----GCAGUCA-3′ are conserved among currently and previously sequenced segments. Oligo-cap analysis showed that the positive strands of the genomic segments are capped, whereas the negative strands are not. Similarities among the four evolutionarily related viruses include low or moderate levels of amino acid sequence identity (14·7–34·2 %) and isoelectric points among equivalent polypeptides, e.g. proteins encoded by segments S4 and S5 of the four viruses. Phylogenetic analysis indicated that MYRV-1/Cp9B21 is related more closely to MYRV-3/RnW370 than to the coltiviruses. An interesting dissimilarity is found in codon-choice pattern among the four viruses, i.e. MYRV-1/Cp9B21 segments have a lower frequency of [XYG + XYC] than corresponding segments of the other viruses, suggesting a possible adjustment of virus codon usage to their host environments.

INTRODUCTION

The seventh report of the International Committee on Taxonomy of Viruses (ICTV) lists nine genera in the large virus family *Reoviridae*, including a wide range of important pathogens of plants, insects, fish and mammals (Mertens *et al.*, 2000). Reoviruses are characterized by distinct properties: (i) they have 10–12 double-stranded (ds) RNA genomic segments, mostly of a monocistronic nature; (ii) virus particles are multi-shelled; and (iii) assembled core particles serve as viral mRNA synthesis factories (reviewed by Nibert & Schiff, 2001). The lack of a reverse genetics system for reoviruses has greatly hampered progress on functional analyses of genome segments. Even mechanical inoculation of plant reovirus particles into cell wall-containing plant hosts has been unsuccessful. Many reoviruses are understood in considerable structural detail: the genera *Orthoreovirus*, typified by *Mammalian orthoreovirus* (Reinisch *et al.*, 2000; Tao *et al.*, 2002), *Orbivirus* (Grimes *et al.*, 1998; Stuart *et al.*, 1998), *Phytoreovirus* (Nakagawa *et al.*, 2003) and *Rotavirus* (Lawton *et al.*, 1997a, b) are good examples. Whilst these viruses have many common structural features, others, including specific viral protein functions, are unique within a particular genus. Therefore, the function of a protein that is encoded by a given dsRNA segment cannot be predicted based on comparison across genera (Mertens *et al.*, 2000).

Recently, three mycoviruses were reported to be tentative or definitive members of the family *Reoviridae*, based upon genomic RNA electropherotype, partial or complete sequences and virion morphology: one from each of two hypovirulent strains (9B21 and C18) of the chestnut blight

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this paper are AB179636–AB179643.

fungus, *Cryphonectria parasitica* (Enebak, 1992; Enebak *et al.*, 1994; Hillman *et al.*, 2004), and one from hypovirulent strain W370 of a root-rot fungus, *Rosellinia necatrix* (Osaki *et al.*, 2002; Wei *et al.*, 2003, 2004). Based on partial sequence and particle properties, a proposal to construct a novel genus, *Mycoreovirus*, in the family *Reoviridae*, with *Mycoreovirus 1* (MYRV1)/Cp9B21, MYRV-2/CpC18 and MYRV-3/RnW370 as members, was recently accepted by the ICTV (Mertens *et al.*, 2004). MYRV-3/RnW370 contains 12 segments of dsRNA that are associated consistently with hypovirulence of that fungus (Osaki *et al.*, 2002). All of the segments have now been sequenced (Osaki *et al.*, 2002; Wei *et al.*, 2003, 2004) and many have been found to be similar to corresponding segments of mammal-pathogenic members, *Colorado tick fever virus* (CTFV) and *Eyach virus* (EYAV), of the genus *Coltivirus* within the family *Reoviridae* (Attoui *et al.*, 2002). Among the genera that are understood less well from the standpoint of particle structure and viral protein function is the genus *Coltivirus*.

The viruses of *C. parasitica* strains 9B21 (MYRV-1/Cp9B21) and C18 (MYRV-2/CpC18), originally isolated from two regions in West Virginia, USA, that were only 20 miles apart, have a double-shelled particle structure that is typical of a reovirus and 11 genomic dsRNA segments (S1–S11) (Enebak, 1992; Enebak *et al.*, 1994; Hillman *et al.*, 2004). The effects of the two viruses on morphology and virulence of their fungal hosts differ somewhat (Enebak, 1992; B. I. Hillman, unpublished results), although detailed studies of their comparative properties have not been performed. Particles of both viruses are infectious when introduced to spheroplasts of *C. parasitica* virus-free strain and induce the same morphological changes that were observed in their original, virus-containing strains (Hillman *et al.*, 2004; B. I. Hillman, unpublished results). Such infectivity is very unusual among mycoviruses.

The sequences of MYRV-1/Cp9B21 segments S1–S3 were determined recently and were found to exhibit approximately 21·7–38·1 % amino acid sequence identity with their counterparts (S1–S3) of MYRV-3/RnW370 and coltiviruses. Although only limited sequence data are available for MYRV-2/CpC18 (R. Festa and B. I. Hillman, unpublished results), the two *C. parasitica* viruses show higher levels of sequence similarity to each other than those between MYRV-1/Cp9B21 and MYRV-3/RnW370.

We now report complete sequences for all the segments of MYRV-1/Cp9B21, which has a unique terminal sequence, 5′-GAUCA-------GCAGUCA-3′, that is strictly conserved in all segments. Based on oligo-cap analysis, positive-sense strands of genomic dsRNA are capped, whereas negative-sense strands are not. Comparative sequence analysis shows interesting similarities and dissimilarities among the four related viruses, MYRV-1/Cp9B21, MYRV-3/RnW370, CTFV and EYAV. Similarities include sizes of the coding regions and amino acid sequences among corresponding segments, providing additional evidence for their molecular evolutionary relatedness. A difference in codon-choice pattern between equivalent segments of the four reoviruses suggests a possible adjustment of codon selection to the nuclear environments of their hosts during the course of co-evolution.

METHODS

Fungal isolates and culturing. Virus-containing isolate 9B21 of *C. parasitica* (Enebak, 1992) was kindly provided by Dr William MacDonald (West Virginia University, Morgantown, WV, USA). This strain was maintained as described by Hillman *et al.* (2004). Virus-infected and uninfected single conidial isolates (9B21 and 9B21ss1) were used in this study. Fungal colonies were grown either on potato dextrose agar (Difco) or in potato dextrose broth (PDB; Difco) under bench-top conditions at 22–26 °C.

dsRNA isolation and cDNA library construction. dsRNA was purified essentially as described previously (Hillman *et al.*, 2004) with minor modifications. Fungal strains were cultured in PDB liquid medium for 1 week at room temperature. Mycelia were harvested on Miracloth (Calbiochem) and homogenized to powder by using a mortar and pestle in the presence of liquid nitrogen. dsRNA was enriched by the use of CC41 cellulose (Millipore) (Isogai *et al.*, 1998), treated with S1 nuclease and subsequently with DNase I. Between nuclease-digestion steps, a round of phenol, phenol/chloroform and chloroform treatment was carried out. After denaturation at 65 °C in 90 % DMSO (Asamizu *et al.*, 1985), total dsRNA was used as a template for cDNA synthesis with random hexamers using a TimeSaver cDNA synthesis kit (Amersham Biosciences). The resulting cDNA was cloned into the *Sma*I site of pBluescript II S+ (Stratagene), which was used for transformation of *Escherichia coli* DH5α. Alternatively, cDNA synthesis was primed on specific dsRNA segments that were isolated with RNaid (Qbiogene) from an agarose gel in 40 mM Tris/acetate, 1 mM EDTA, pH 7·8 (TAE), as described by Hillman *et al.* (2004), and cloned into the vector pGEM-T Easy (Promega).

Terminal sequence determination. The extreme termini of the genome segments were determined by three methods. For a classic 5′-RACE (rapid amplification of cDNA ends) protocol, approximately 20 ng purified dsRNA, along with specific primers, was denatured in 90 % DMSO as described by Asamizu *et al.* (1985) and precipitated in ethanol. Sequences of the primers used for the respective segments are available upon request. The precipitate was suspended with 25 µl pre-warmed (42 °C) reverse transcription reaction mixture [50 mM Tris/HCl (pH 8·3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT], 50 U MMLV reverse transcriptase (Fermentas) and 20 U RNase inhibitor (Toyobo). As described by Polashock & Hillman (1994), cDNA products were used as template with the 5′ abridged anchor primer and a nested specific primer after d(C) tailing or d(A)–d(C) tailing with terminal deoxynucleotidyltransferase.

For RNA ligase-mediated RACE (RLM-RACE) (Maruyama & Sugano, 1994), purified dsRNA was treated with calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP), which removed the cap structure, and then denatured in DMSO as described above. The oligoribonucleotide 5′-GCUGAUGGCGAUGAAUGAACACUGCG-UUUGCUGGCUUUGAUGAAA-3′ was ligated to the to the 5′ termini of denatured genomic RNA. cDNA was synthesized with random decamers and amplified according to the protocol provided in the Ambion First Choice RLM-RACE kit.

Additionally, a modified oligodeoxynucleotide-mediated RT-PCR protocol (Lambden *et al.*, 1992) was employed to determine terminal sequences of S11. A 5′-phosphorylated, 3′-amino-linked oligodeoxynucleotide (5′-PO_4-CACTGCGTTTGCTGGCTTTGG-NH_2-3′) was ligated to each of the 3′ termini of the MYRV-1/Cp9B21 genomic segment with T4 RNA ligase at 4 °C for 16 h. Ligated S11 segments,

isolated from 1 % agarose gels, were denatured at 65 °C for 15 min in 90 % DMSO and used as templates for cDNA synthesis with an oligonucleotide complementary to the modified one. Following repair with Klenow fragment (Toyobo), the resulting cDNA was amplified by PCR with the single primer complementary to the adaptor. Amplified DNA fragments were cloned into the pGEM-T Easy vector.

Sequencing and sequence analysis. Plasmid DNA, prepared by using Qiagen spin columns, was sent to Macrogen (Seoul, Korea) for sequencing on an ABI PRISM 3700 DNA analyser. Alternatively, sequencing was performed on an ABI model 2100 or model 377 sequencer. Sequences were analysed with the aid of the GENETYX DNA-processing software (SDC). Database searches were performed with the BLAST 2.0 program (Altschul *et al.*, 1997) from the National Center for Biotechnology Information (NCBI), the FASTA 3 program (Pearson & Lipman, 1988) from the DNA Database of Japan (DDBJ) or the FASTA (Lipman & Pearson, 1985) program in the GENETYX DNA-analysing software suite.

RNA blot analysis. Northern blot analysis was as described previously (Suzuki *et al.*, 2003). Total genomic dsRNA was separated by electrophoresis through 1·2 % agarose gels under denaturing conditions. Fractionated RNA was capillary-transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences) and probed with digoxigenin (DIG)-11-dUTP-labelled DNA fragments that were amplified by PCR according to the method recommended by the manufacturer (Roche Diagnostics). Chemiluminescent signals of probe–RNA hybrids were detected by using a DIG detection kit and a CDP star kit (Roche) and then visualized in a Hamamatsu Photonics real-time image processor (model Argus-50).

RNA dot-blot analysis was carried out as described previously (Suzuki *et al.*, 1990). Genomic segments S7 and S8 were isolated after SDS-PAGE and denatured for 5 min at 65 °C in 1× MOPS (20 mM morpholinopropane/sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7·0), 50 % formamide, 16 % formaldehyde. After chilling on ice, the denatured dsRNA was dot-blotted onto a Hybond-N membrane (Amersham Biosciences) and probed with DIG-labelled cDNA of specific segments as described above. Segments S5 and S6 were resolved through long agarose gels as described below, then excised, blotted and probed with ^{32}P-labelled cDNA.

RESULTS

Assignment of cDNA clones to MYRV-1/Cp9B21 genomic segments

In keeping with current reovirus segment nomenclature, genomic segments of MYRV-1/Cp9B21 were termed S1–S11 with increasing order of gel mobilities. Order of migration of the segments was the same in agarose and acrylamide gels, although acrylamide gel gave better resolution. The three largest of the 11 genomic RNA segments (S1–S3) of MYRV-1/Cp9B21 were sequenced previously (Hillman *et al.*, 2004). Thus, in this study, the remaining, smaller segments were targeted. From cDNA libraries derived from total genomic dsRNA, cDNA clones with relatively large inserts of over 400 bp were chosen randomly. Genomic segments S4, S5 and S6, purified from agarose gels, were also used as templates in cDNA synthesis as described by Hillman *et al.* (2004). Assembly of sequences of cDNA clones to total dsRNA segments and gel-isolated segments resulted in a total of 11 contigs, three of which represented S1–S3. Single cDNA clones representing each of the other eight contigs were labelled with DIG and used as probes for Northern blots.

As shown in Fig. 1, each probe detected a single band on a Northern blot (lanes 4–11). It was difficult to distinguish S5 from S6 and S7 from S8, as they were of similar size and co-migrated on this gel system. Therefore, segments S5 and S6 were resolved by electrophoresis for 28 h through a 23 cm, 1·4 % agarose gel run in TBE (89 mM Tris/borate, 89 mM boric acid, 2 mM EDTA, pH 8·0). By this time, segments S5 and S6 had migrated approximately 18 cm and were resolved into two segments that could be excised with a razor blade, purified by using GeneClean and examined by dot-blotting and hybridization with [^{32}P]-labelled probes that were amplified from segment S5 (nt 1423–1822) or S6 (nt 317–809). Consequently, it was shown that, as expected from their length in nucleotides, contigs 5 and 6 were assigned to S5 and S6, respectively (data not shown). Similarly, genome segments S7 and S8 were separated by SDS-PAGE, dot-blotted and subjected to hybridization and DIG detection. This resulted in the assignment of contigs 7 and 8 to S8 and S7, respectively. A non-size-based separation on polyacrylamide gels is often found for reoviral dsRNA segments (Suzuki *et al.*, 1990), but no such migration pattern was observed for the MYRV-1/Cp9B21 segments.

Terminal structures

Classic 5′-RACE was used to determine the terminal sequence of the segments, as used for S1–S3 of MYRV-1/Cp9B21. Most 5′-RACE clones of positive strands of

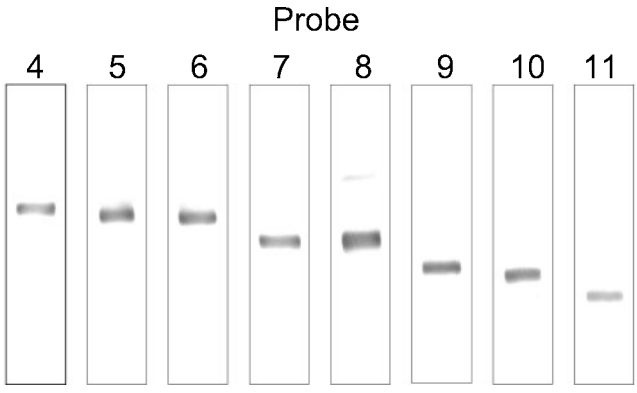


Fig. 1. Assignment of cDNA contigs to genomic dsRNA segments of MYRV-1/Cp9B21. After denaturation in 3·7 % formaldehyde and 42·5 % formamide at 65 °C for 15 min, purified 9B21 dsRNA was separated by 1·4 % agarose gel electrophoresis under denaturing conditions and capillary-transferred onto a nylon membrane. The membrane was stripped into eight pieces and each was probed by DIG-labelled PCR fragments representing cDNA contigs (4–11). cDNA fragments used as probes spanned map positions 1231–2013, 522–1124, 413–962, 931–1536, 1–1539, 459–1072, 107–513 and 309–712 for contigs 4–11, respectively.

genome segments were 5′-GATCA---, whereas some lacked several 5′ terminal nucleotides. The 5′ sequence GAUCA was confirmed by sequencing RLM-RACE clones. The negative strands of genome segments were also determined by classic RACE analysis to be 5′-UGACUGC---. The 5′ pentamer, 5′-GAUCA---, and 3′ octamer, ---CGCAGUCA-3′, are conserved among the three largest segments of MYRV-1/Cp9B21 (Hillman *et al.*, 2004). However, the eighth residue from the 3′ end was not shared in S5, S7, S9 or S11, as summarized in Fig. 2a. As reported by Hillman *et al.* (2004), the 3′ end is similar to those of MYRV-3/RnW370 (---UGCAGAC-3′) and coltiviruses (---a/uUGyAGUg/c-3′), whereas the 5′ terminus is similar to that of members of the genus *Oryzavirus* (5′-GAU---) or coltiviruses (5′-GA---).

The oligo-cap method (RLM-RACE), which entailed RNA ligase-mediated adaptor-oligonucleotide ligation to the 5′ termini and RT-PCR, was used to examine whether the 5′ termini of genome segments were capped (blocked). Three different preparations of dsRNA segments (those with no treatment, those treated with CIP only and those treated with CIP and TAP) were subjected to adaptor ligation and RT-PCR. As shown at the bottom of Fig. 3, these preparations provide different RT-PCR amplification profiles, depending on the structure of genome segments. Ligatable RNA with free 5′ phosphate groups can lead to the production of amplified DNA fragments of the expected size, whereas a segment with 5′ hydroxyl groups or covalently genome-linked viral protein cannot produce PCR fragments in any preparation. If the 5′ terminus of a segment is capped, PCR fragments should be obtained only on CIP/TAP-treated templates. No PCR products will be obtained on CIP-treated cDNA, irrespective of whether the MYRV-1/Cp9B21 genomic segments are capped.

As an example of oligo-cap reactions, results with S10 and S11 are shown in Fig. 3. For the 5′ end of the S10 positive strand, a DNA fragment of approximately 320 bp was generated on untreated and CIP/TAP-treated preparations (Fig. 3, S10+, lanes 1 and 3). A PCR band of the expected size (190 bp) was observed on untreated samples for the 5′ end of the S10 negative strand (Fig. 3, S10−, lane 1), whereas no band of the expected size was found on CIP- or CIP/TAP-treated dsRNA. Nevertheless, minor bands of smaller sizes than expected were detected (Fig. 3, S10−, lanes 2 and 3). A similar oligo-cap profile was found for S11 (Fig. 3, S11). DNA fragments of approximately 300 bp, covering the 5′ terminus of the S11 positive strand, were produced in untreated and CIP/TAP-treated dsRNA

(a)

```
      S1 5′-GAUCAAUACACGAAAUUAAG------GCAGUGGUGUAUCGCAGUCA-3′
      S2 5′-GAUCAAUACUUCAUAACUAC------GUGUUUCGGUAUCGCAGUCA-3′
      S3 5′-GAUCAUUUUUCACUCACUAC------ACACACCACGCCCGCAGUCA-3′
      S4 5′-GAUCAAAUAATTCAACATAC------TGCTCTGGATTACGCAGUCA-3′
      S5 5′-GAUCAAUUUCUCAUACUUAC------GGGAAACCGUAUAGCAGUCA-3′
      S6 5′-GAUCAAAAGUACUGAAUAUG------GCGUCGUACUCCCGCAGUCA-3′
      S7 5′-GAUCAAUUUAAAGCCUAUAG------AAGGCUAGGAUGCGCAGUCA-3′
      S8 5′-GAUCAAUAUCGCACGCAAAC------ACCGAGUCGAUAUGCAGUCA-3′
      S9 5′-GAUCAUUUGAGUACCUAAAC------UGUUAAUCGAAUUGCAGUCA-3′
     S10 5′-GAUCAAAAAUAACGAUAUAC------AGUGCCGUUAUACGCAGUCA-3′
     S11 5′-GAUCAUAAUAUCCUGUAUAC------GAGAUGAUGUAUUGCAGUCA-3′
Consensus 5′-GAUCAa/ua/ua/u-------------------------GCAGUCA-3′

   MYRV3 5′-ACAAUUUu/au/aa/ga/g-------------------UGCAGAC-3′
    CTFV 5′-g/cACAUUUUG-----------------------a/uUGCAGUg/c-3′
    EYAV 5′-GACAa/uUUg/u-------------------------a/uUGyAGUC-3′
```

(b)

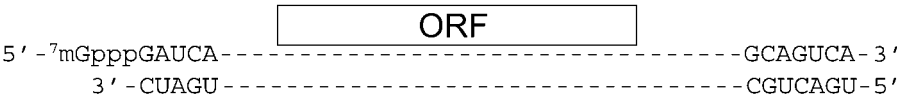
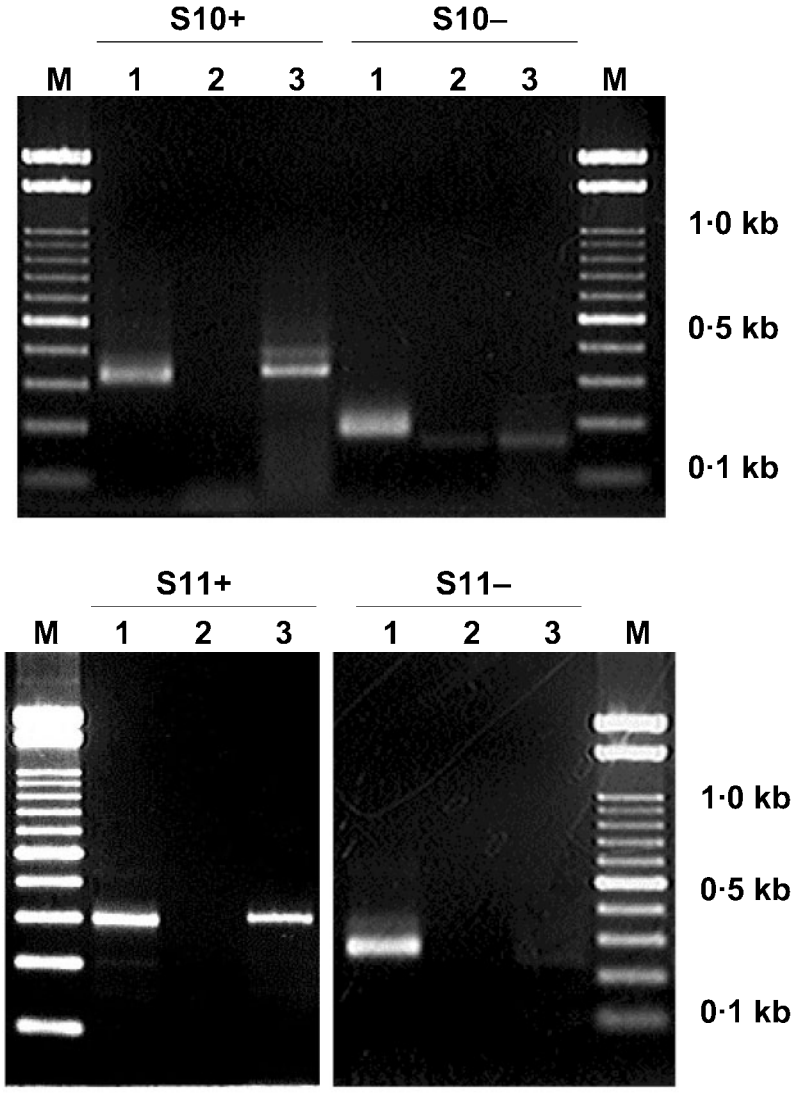


Fig. 2. Terminal sequences and basic structure of the MYRV-1/Cp9B21 genome segments. (a) Both terminal sequences (20 nt) of the ORF-containing strand of each segment were aligned from the end base. Highly conserved consensus sequences are shown below. The sequences of MYRV-1/Cp9B21 segments S1–S3 were reported previously (Hillman *et al.*, 2004). The terminal sequences of related viruses (MYRV-3/RnW370, EYAV and CTFV) are also indicated. (b) The basic structure of the MYRV1/Cp9B21 genome segments (conserved 5′ and 3′ nucleotide sequences and coding domain) is shown in a schematic form.



Terminal structure	Treatment		
	None (lane 1)	CIP (lane 2)	CIP+TAP (lane 3)
Cap	–	–	+
VPg	–	–	–
Phosphate	+	–	–
Hydroxyl	–	–	–

Fig. 3. Oligo-cap analysis of MYRV-1/Cp9B21 genomic segments. Purified genomic dsRNA was left untreated or treated with CIP and subsequently with TAP (a decapping enzyme) after denaturation with DMSO at 65 °C for 15 min. The untreated (lane 1), CIP-treated (lane 2) and CIP plus TAP-treated (lane 3) RNA preparations were ligated to an oligoribonucleotide (see Methods for sequence) at their 5′ termini, then subjected to cDNA synthesis with random decamers. cDNA covering the 5′ region of S10 and S11 was amplified by PCR using a set of primers: one specific primer for one of the MYRV-1/Cp9B21 segments and an anchor primer that was homologous to the adaptor sequence. The segment-specific primers used were 9B10-3 (map positions 269–287) for the S10 positive strand, 9B10-2 (map positions 832–850) for the S10 negative strand, 9B83 (map positions 247–264) for the S11 positive strand and 9B100 (map positions 506–526) for the S11 negative strand. Amplified fragments were electrophoresed in 1·8% agarose gel in a TBE buffer system. Expected sizes of PCR fragments are 322 bp for the S10 positive strand, 188 bp for the S10 negative strand, 309 bp for the S11 positive strand and 271 bp for the S11 negative strand. Expected oligo-cap reaction patterns are shown for four possible terminal structures of a viral RNA segment at the bottom. Expectations of positive and negative reactions are denoted + and –, respectively.

preparations (lanes 1 and 3). Only untreated preparations generated a fragment of 270 bp, which was expected from the 5′ terminal sequence of S11 negative strands.

These oligo-cap analyses clearly indicated the presence and absence of cap structures on the positive and negative strands of MYRV1/Cp9B21 genomic RNA, respectively. However, the fact that both untreated and CIP/TAP-treated samples produced RT-PCR fragments for the S10 and S11 positive strands may suggest that some MYRV1 genomic RNA in infected *C. parasitica* cells is capped, whereas some is uncapped. In this regard, it is interesting to note that the 5′ termini of CIP-treated positive strands of *Rice gall dwarf virus* (a phytoreovirus) can also be labelled with [γ-^{32}P]ATP and polynucleotide kinase, suggesting the existence of free phosphates at their 5′ ends (Kudo *et al.*, 1991). An alternative interpretation may be that the capped genomic positive-sense strands are partially digested at their 5′ termini to have free phosphates during RNA isolation.

Sequences of MYRV-1/Cp9B21 segments S4–S11

The complete sequence of each segment was obtained from cDNA clones and 5′-RACE clones. As summarized in Table 1, the lengths of the MYRV-1/Cp9B21 genome

Table 1. Properties of CpMYRV1 genome segments

Sequence data on MYRV-1/Cp9B21 S1–S3 in this and subsequent tables are from Hillman *et al.* (2004).

Segment	Size (nt)	Protein	M_r (size in aa)	Counterpart		Remark	GenBank accession no.
				MYRV3	Coltivirus		
S1	4127	VP1	151 780 (1354)	P1	VP1	RdRp	AY277888
S2	3846	VP2	137 560 (1238)	P2	VP2		AY277889
S3	3258	VP3	120 823 (1065)	P3	VP3	Similar to BmCPV VP3	AY277890
S4	2269	VP4	79 837 (720)	P4	VP4	Myristoylated	AB179636
S5	2056	VP5	72 764 (647)	P5	VP5		AB179637
S6	2023	VP6	73 418 (649)	P6	VP10	NTP-binding	AB179638
S7	1539	VP7	51 179 (469)	P9		Proline-rich, leucine zipper	AB179639
S8	1536	VP8	54 093 (481)	P7			AB179640
S9	1072	VP9	32 880 (297)	P11			AB179641
S10	975	VP10	27 758 (247)				AB179642
S11	732	VP11	11 461 (101)				AB179643

segments range from 4127 (S1) to 732 (S11) bp, which are in accord with the agarose gel-electrophoresis profile (Hillman *et al.*, 2004). All of the segments except for S11 possess single, large ORFs that span >90 % of their entire segment sizes. S11 contains only a relatively small ORF that corresponds to <50 % of the entire segment sequence. Small ORFs comprising more than 300 nt, as well as the large ORFs, are found on the positive strands of S2 and S4 and the negative strands of S1 and S9, but it remains to be answered whether these small ORFs are expressed. As is the case with other reoviruses, each segment contains common terminal sequences. The 5′ conserved pentanucleotide of MYRV-1/Cp9B21 is 5′-GAUCA--- and the 3′ heptanucleotide is ---GCAGUCA-3′ (Fig. 2a). Nucleotides at positions 6–8 from the 5′ end are semi-conserved (a/u-a/u-a/u). As reported for MYRV-1/Cp9B21 S1–S3 (Hillman *et al.*, 2004), no typical inverted repeat structure, which is found in plant reoviruses (Xu *et al.*, 1989; Kudo *et al.*, 1991), was identified from the conserved terminal sequences or adjacent residues of segments S4–S11.

Interviral amino acid sequence similarities and amino acid sequence motifs

Osaki *et al.* (2002), Wei *et al.* (2003, 2004) and Hillman *et al.* (2004) noted the relatedness of four viruses: two fungus-infecting viruses, MYRV-1/Cp9B21 and MYRV-3/RnW370, and two members of the genus *Coltivirus* (CTFV and EYAV). A BLAST search revealed similarities among overall sequences of VP1 proteins encoded by the largest segments of the viruses (37 % identity for MYRV-1/Cp9B21 vs MYRV-3/RnW370, 29 % for MYRV-1/Cp9B21 vs EYAV, 28 % for MYRV-1/Cp9B21 vs CTFV) and among VP2 proteins (28, 22 and 22 % for the same order of pairwise comparisons, respectively) (Table 2). Sequence similarities were found between limited regions of S3-encoded proteins of MYRV-1/Cp9B21 and the other three viruses.

Sequence database searches were performed with the newly and previously determined sequences of MYRV1/Cp9B21 S1–S11 by using the BLAST and FASTA programs. Sequence similarities and optimal scores among the four viruses are summarized in Table 2. In addition to the previously identified equivalent segments, a BLAST search identified homologous segments, including MYRV-1/Cp9B21 S4, MYRV-3/RnW370-S4 and coltivirus S4, MYRV-1/Cp9B21 S6, MYRV-3/RnW370 S6 and coltivirus S10 and MYRV1/Cp9B21 S9 with MYRV-3/RnW370 S11. FASTA 3 searches further identified MYRV-1/Cp9B21 S5 and MYRV-3/RnW370 S5. Most pairwise alignments created for MYRV-1/Cp9B21 and MYRV-3/RnW370 by FASTA 3 are continuous, extending from near the N termini to the C termini (see overlaps in Table 2), whereas BLAST alignments are shorter than FASTA alignments and are discontinuous or localized for some segments, such as S4 (data not shown). MYRV-1/Cp9B21 S7 and S8 did not yield any significant hit in BLAST or FASTA 3 searches. However, by using FASTA in the GENETYX suite, low sequence identities (14·7 and 15·7 %) were detected between MYRV-1/Cp9B21 S7 and MYRV-3/RnW370 S9 and between MYRV-1/Cp9B21 S8 and MYRV-3/RnW370 S7, respectively. The sequence similarities, as well as analogous isoelectric points (p*I*) (5·54 vs 4·90 and 5·74 vs 5·40, respectively; see Table 2), suggest that they are homologous to each other. It remains unclear which segments of MYRV-3/RnW370 and coltiviruses are counterparts of MYRV-1/Cp9B21 S10 and S11, as these show no significant similarities to other known sequences.

The two largest segments of the four viruses are more similar than any other corresponding segments and similarities between corresponding segments of MYRV-1/Cp9B21 and coltiviruses are smaller in scope than those between the two mycoviruses (Table 2). Multiple sequence alignments using CLUSTAL W (Thompson *et al.*, 1997) revealed several relatively well-conserved regions that included the N-terminal 100 aa and middle portion of VP4 proteins of the four viruses, and C-terminal half of

Table 2. Similarities among related reoviruses

Segment*			Non-coding (nt)						Coding capacity (aa)			p*I*			Amino acid sequence similarity†					
Cp	Rn	Ey	5′			3′			Cp	Rn	Ey	Cp	Rn	Ey	Cp/Rn			Cp/Ey		
			Cp	Rn	Ey	Cp	Rn	Ey							Identity (%)	Overlap (aa)	Opt	Identity (%)	Overlap (aa)	Opt
(S1,	S1,	S1)	25	28	12	37	32	29	1354	1360	1435	7·92	7·91	7·62	38·1	1376	2268	29·9	1342	1369
(S2,	S2,	S2)	80	62	44	49	30	62	1238	1226	1275	6·43	6·05	5·92	30·5	1241	1996	23·3	1260	1050
(S3,	S3,	S3)	27	9	10	33	40	26	1065	1086	1182	7·21	6·31	6·18	21·7	1117	461	24·8	105	97
(S4,	S4,	S4)	38	28	20	68	53	52	720	725	1027	6·35	6·02	6·00	22·4	675	297	34·2	111	173
(S5,	S5,	S5)	42	45	68	70	103	74	647	646	751	5·96	6·43	6·05	19·7	612	249	22·1	398	131
(S6,	S6,	S10)	17	95	10	56	30	51	649	624	605	8·83	8·44	8·87	27·4	540	649	23·2	224	199
(S7,	S9,	?)	50	45	–	79	38	–	469	380	–	5·74	5·40	–	14·7	333	92			
(S8,	S7,	?)	39	19	–	51	41	–	481	482	–	5·54	4·90	–	15·7	421	83			
(S9,	S11,	?)	87	81	–	91	73	–	297	276	–	5·71	8·15	–	21·2	179	153			

*Cp, Rn and Ey refer to MYRV-1/Cp9B21, MYRV-3/RnW370 and EYAV, respectively.

†Amino acid sequence similarities were determined by FASTA 3 (Pearson & Lipman, 1988) at DDBJ, except those between MYRV-1/Cp9B21 S7 and MYRV-3/RnW370 S9 and between MYRV-1/Cp9B21 S8 and MYRV-3/RnW370 S7, which were calculated by FASTA (Lipman & Pearson, 1985) in the SDC GENETYX suite. GenBank accession numbers are AB102674, AB098022, AB102675, AB073276, AB098023 and AB073277–AB073283 for MYRV-3/RnW370 S1–S12, and NC_003696–NC_003707 for EYAV S1–S12, respectively. GenBank accession numbers of the MYRV-1/Cp9B21 segments are shown in Table 1.

VP6 proteins of the two mycoviruses and VP10 proteins of coltiviruses (see below).

Amino acid sequence similarities were also found between MYRV-1/Cp9B21 and viral sequences other than the related reoviruses mentioned above. MYRV-1/Cp9B21 VP3 shows sequence similarity (identity, 21·8 %; optimal score, 158; overlap, 829 aa) to outer capsid protein VP3 encoded by *Bombix mori* cypovirus 1 S4 (Ikeda *et al.*, 2001). MYRV-1/Cp9B21 S8 exhibited amino acid sequence similarity (identity, 28·1 %; optimal score, 223; overlap, 242 aa) to *Turnip yellow mosaic virus* RNA-dependent RNA polymerase (RdRp) protein. Other similarities identified by FASTA 3 included those found between MYRV-1/Cp9B21 S6 and a *Nilaparvata lugens virus* (NLRV) inner core protein with the purine-binding motif (Nakashima *et al.*, 1996) (identity, 22·1 %; optimal score, 110; overlap, 340 aa). Such a domain was recently described as being unique to turreted members of the family *Reoviridae* and coltiviruses (Nibert & Kim, 2004). Both motifs A and B described in that study were identified in MYRV-1/Cp9B21 (Fig. 4b). The NTP-binding-like motif A/GXKXXGK at residues 438–444 of MYRV-1/Cp9B21 VP4, and a well-conserved stretch downstream of the motif at residues 458–465 of MYRV-1/Cp9B21 VP4, are found among MYRV-1/Cp9B21, MYRV-3/RnW370, two coltiviruses and many fijiviruses, as noted by Attoui *et al.* (2002) and McQualter *et al.* (2003). Whether the purine-binding motif serves as a real NTP-attachment site has yet to be determined. The adjacent conserved region, DSDXY/FG, termed motif 2 by McQualter *et al.* (2003), may be important as part of the binding domain or for independent function.

The programs MYRbase (Maurer-Stroh *et al.*, 2004) and Myristoylator (Bologna *et al.*, 2004) both predicted that VP4 of MYRV-1/Cp9B21 was myristoylated at the N terminus. As MYRV-3/RnW370 VP4 and coltivirus VP4 proteins were all predicted to be homologues of VP4 of MYRV-1/Cp9B21, all were examined for possible myristoylation. Visual inspection revealed that the protein deduced from MYRV-3/RnW370 S4 was not predicted to be myristoylated; this was confirmed by both programs. All of the other VP4 sequences (Fig. 4a), as well as the N-terminal region of MYRV-2/CpC18 VP4 (R. Festa and B. Hillman, unpublished results), were strongly predicted to be myristoylated.

No other sequence motif was observed except for the NTP-binding motif of VP6, RdRp of VP1 and relatively commonly found motifs, such as the proline-rich (aa residues 334–421) and leucine zipper (aa residues 125–146 and 132–153) regions, which were found for S8 (Table 1).

Comparative sequence analyses of the four related reoviruses

It is now clear that most segments of MYRV-1/Cp9B21 correspond to those of MYRV-3/RnW370, EYAV and CTFV. Equivalent segments of the four viruses were further compared in sizes of coding and non-coding sequences, p*I* values and codon-choice pattern. Comparative sequence analysis revealed a similarity in the length of coding sequences, as well as of the 5′ non-coding region (NCR), between the corresponding segments, particularly of the two mycoviruses, as shown in Table 2. The sizes of the 5′ NCRs of MYRV-1/Cp9B21 S3 and S8 and

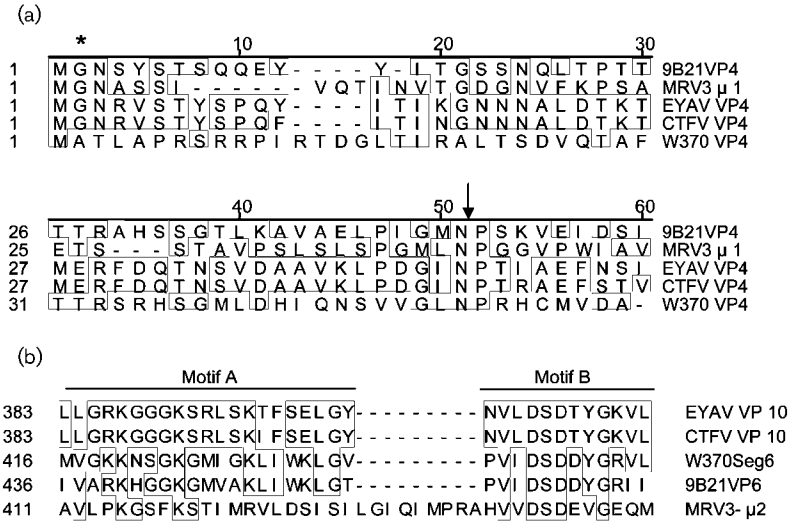


Fig. 4. Conserved motifs in MYRV-1/Cp9B21 VP4 and VP6. (a) Comparison of the deduced N-terminal regions of VP4 sequences of MYRV-1/Cp9B21 (GenBank accession no. AB179636), MYRV-3/RnW370 (BAC07518), EYAV (AAM18345) and CTFV (AAG00069) with the N-terminal sequence of the μ1 protein of MRV-3 (AAA47258.1). The asterisk denotes the putatively myristoylated G residue and the arrow denotes the NP dipeptide that is present in all sequences examined and known to be critical to proteolytic cleavage of the MRV-3 μ1 protein. (b) Alignment of sequences representing conserved motifs A and B of the NTP-binding domains of VP6 of MYRV-1/Cp9B21 (AB179638) and MYRV-3/RnW370 (BAC07519), VP10 of EYAV (AAM18352) and CTFV (AAG00074) and μ2 protein of MRV-3 (AAL99937).

MYRV-3/RnW370 S6, and the lengths of coding regions of MYRV-1/Cp9B21 S8 and EYAV S4, differ from those of their counterparts. The size of the 3′ non-translatable sequence does not vary greatly among equivalent segments, with a range of 33–103 bp. The p*I* values of deduced proteins of equivalent segments are also comparable, with the difference among them being $\pm$10 %, except for the values of 5·71 and 8·15 for MYRV-1/Cp9B21 VP9 and MYRV-3/RnW370 P11. Phytoreovirus segments of the same origin were shown to have similar lengths of 5′ NCR, but not 3′ NCR (Xu *et al.*, 1989; Suzuki *et al.*, 1990). The similarity in the lengths of 5′ NCR of corresponding segments is greater in phytoreoviruses than in MYRV-1/Cp9B21 and related viruses.

Comparative codon-choice analysis showed an interesting difference among equivalent segments of the three viruses. Codon-selection patterns for several amino acids are shown in Table 3. For example, the GUG codon for Val is used least frequently in MYRV-1/Cp9B21, but is the most or second most frequently used codon in EYAV, whereas the GUA codon for Val is the least frequently selected in MYRV-3/RnW370. The frequencies of codons GGC and GGG for Gly are much lower than those of GGU and GGA in MYRV-1/Cp9B21, whereas the GGC and GGG codons are used relatively often in MYRV-3/RnW370 and EYAV.

As expected from the above examples, when compared between counterparts, MYRV-1/Cp9B21 segments have a higher frequency of [XYU] and [XYA] and a lower frequency of [XYG] than corresponding segments of MYRV-3/RnW370 or coltiviruses. For instance, the frequency of [XYU] in VP1 proteins is 41·2 % for MYRV-1/Cp9B21, 30·3 % for MYRV-3/RnW370 and 34·9 % for EYAV (Table 4). When compared among the corresponding segments, MYRV-3/RnW370 and coltiviruses have the highest and second highest frequency of [XYG+XYC], whereas the frequency of [XYG+XYC] in MYRV-1/Cp9B21 is the lowest. The difference in the frequency of [XYG+XYC] ranges from 19·2 % (MYRV-1/Cp9B21 S1 vs MYRV-3/RnW370 S1) to 4·6 % (MYRV-1/Cp9B21 S9 vs MYRV-3/RnW370 S11).

DISCUSSION

The completion of sequence analysis of a mycovirus (MYRV-1/Cp9B21) that occurs naturally in a hypovirulent strain (9B21) of the chestnut blight fungus, *C. parasitica*, reveals features of this virus that are characteristic of reoviruses and strengthens a suggestion made by Enebak *et al.* (1994), Osaki *et al.* (2002) and Hillman *et al.* (2004) that the MYRV-1/Cp9B21-related mycoviruses comprise a novel genus within the family *Reoviridae*. The ICTV recently approved the genus *Mycoreovirus* within the family *Reoviridae*. The novel genus contains three species: MYRV-1/Cp9B21, *C. parasitica* mycoreovirus 2/C18 (MYRV-2/CpC18) and *R. necatrix* mycoreovirus 3/W370 (MYRV-3/RnW370). Therefore, the family *Reoviridae* now accommodates 11 genera, with a large number of members that infect fungi, plants, invertebrates and vertebrates.

The conserved terminal sequences and the number of genome segments are very important criteria for reovirus taxonomy (Mertens *et al.*, 2000). The genus *Mycoreovirus* is different from most other genera in that it contains species with different numbers of genome segments: 11 for MYRV-1/Cp9B21 and MYRV-2/CpC18 and 12 for MYRV-3/RnW370. A hint to the paradox of the genome segment number may come from the evolutionary scenario that was proposed by Hillman *et al.* (2004), i.e. mycoreoviruses and coltiviruses may have diverged from a progenitor virus that infected an Acari arthropod species and had 12 genome segments. During the course of evolution, the *C. parasitica* mycoreoviruses may have lost a segment that was required

Table 3. Codon-choice pattern for Leu, Val, Pro, Thr and Gly

Codon	S1*			S2			S3			S4			S5			S6 (S10 in Ey)		
	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey
UUA-Leu	42	19	41	18	17	25	32	19	43	20	6	24	12	4	8	18	9	18
UUG-Leu	7	25	40	17	33	32	17	17	29	8	16	18	7	12	20	11	14	21
CUU-Leu	38	33	30	40	9	20	31	31	19	17	10	16	20	10	17	8	9	20
CUC-Leu	15	16	9	14	30	16	10	17	11	4	17	6	12	11	8	10	10	7
CUA-Leu	20	14	11	6	9	12	17	10	6	5	6	8	7	4	4	9	7	7
CUG-Leu	7	13	19	15	15	19	5	13	17	3	6	16	2	2	12	6	10	6
GUU-Val	32	56	41	42	41	39	32	44	45	25	36	31	19	26	20	25	23	23
GUC-Val	17	22	11	27	28	13	12	29	11	6	19	9	16	15	10	12	13	3
GUA-Val	20	7	23	26	9	21	11	13	18	12	10	15	6	3	7	6	11	7
GUG-Val	8	15	26	6	20	36	22	18	31	4	14	35	8	12	21	7	13	8
CCU-Pro	24	15	23	27	14	20	22	20	23	12	9	19	13	7	16	10	5	11
CCC-Pro	4	14	6	29	27	7	11	10	7	3	8	2	4	10	10	7	7	1
CCA-Pro	36	19	16	22	17	26	25	17	10	16	13	10	16	11	13	15	4	6
CCG-Pro	12	18	13	9	13	15	9	10	7	4	9	8	4	5	9	5	12	2
ACU-Thr	42	21	24	48	16	18	29	21	13	18	13	18	21	9	10	20	10	22
ACC-Thr	17	17	9	19	21	11	17	10	5	6	13	6	9	15	4	5	10	6
ACA-Thr	30	28	16	22	25	13	24	9	9	21	16	24	12	11	9	15	8	6
ACG-Thr	12	25	24	15	16	16	9	16	15	8	11	14	2	7	13	5	19	4
GGU-Gly	38	25	36	31	33	27	16	27	29	14	17	25	14	16	15	18	9	16
GGC-Gly	7	24	12	6	14	12	5	12	10	9	15	9	3	11	11	2	6	7
GGA-Gly	33	24	31	25	18	21	13	18	23	16	15	32	9	14	19	9	8	15
GGG-Gly	4	19	24	1	9	21	1	5	21	4	12	27	3	7	12	4	7	9

*Cp, Rn and Ey refer to MYRV-1/Cp9B21, MYRV-3/RnW370 and EYAV, respectively.

in an arthropod or other host, but was not required in the fungal host. In this regard, it is of great interest that MYRV-3/RnW370 can replicate in its host even after the complete loss of the eighth-smallest segment during passage in the laboratory (Kanematsu *et al.*, 2004).

At least a few of the most terminal sequences are identical among species of a genus in the family *Reoviridae*. For example, 5′-GGXA----UGAU-3′ is conserved in all three members of the genus *Phytoreovirus*, whereas 5′-GUU---UAC-3′ is highly conserved in the genus *Orbivirus*.

Table 4. Comparative codon-usage analysis

Segment*			[XYU] (%)			[XYC] (%)			[XYA] (%)			[XYG] (%)			[XYG+XYC] (%)		
Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey	Cp	Rn	Ey
S1,	S1,	S1	41·2	30·3	34·9	14·1	22·4	13·0	30·5	22·2	23·2	14·2	25·1	28·9	28·3	47·5	41·9
S2,	S2,	S2	40·8	29·7	36·7	22·0	28·7	14·6	24·3	18·7	21·1	12·9	22·8	27·7	34·9	51·5	42·2
S3,	S3,	S3	41·8	35·3	38·1	19·2	24·3	12·4	26·0	19·7	22·7	13·0	20·6	26·8	32·2	44·9	39·2
S4,	S4,	S4	43·6	34·3	35·8	14·6	22·5	11·7	30·5	20·4	23·8	11·4	22·9	28·7	25·9	44·3	40·3
S5,	S5,	S5	39·8	31·1	30·6	22·4	30·1	19·8	24·9	16·7	19·8	13·0	21·9	29·8	35·3	52·0	49·6
S6,	S6,	S10	36·6	30·4	40·8	16·8	23·9	13·0	30·3	19·8	21·6	16·3	25·8	24·6	33·1	49·8	37·6
S7,	S9	–	42·3	29·1	–	21·9	27·3	–	22·6	22·6	–	13·2	21·0	–	35·1	48·3	–
S8,	S7	–	41·7	32·1	–	17·4	22·6	–	27·0	24·6	–	13·9	20·7	–	31·3	43·3	–
S9,	S11	–	32·2	33·9	–	28·9	28·3	–	26·5	20·1	–	12·4	17·7	–	41·3	45·9	–
Host			Cpr	Rnc	Hsp	Cpr	Rnc	Hsp	Cpr	Rnc	Hsp	Cpr	Rnc	Hsp	Cpr	Rnc	Hsp
			30·2	18·1	21·9	28·4	37·8	30·2	21·3	15·6	19·2	20·8	28·5	28·7	49·2	66·3	58·9

*Cp, Rn and Ey refer to MYRV-1/Cp9B21, MYRV-3/RnW370 and EYAV, respectively. Data on codon usage in genomes of *C. parasitica* (Cpr), *R. necatrix* (Rnc) and *H. sapiens* (Hsp) are from http://www.kazusa.or.jp/codon/.

However, the terminal sequences are different between the two mycoreoviruses; 5′-GAUAC----GCAGUCA-3′ for MYRV-1/Cp9B21 and 5′-ACAAUU----GCAGAC-3′ for MYRV-3/RnW370, although the tetranucleotide ---GCAG--- at positions 7–4 and 6–3 from the 3′ ends of MYRV-1/Cp9B21 and MYRV-3/RnW370, respectively, are shared. A very similar case is that of the genus *Cypovirus*, which currently includes definitive and putative members with differing terminal sequences and numbers of genomic segments (Rao *et al.*, 2003).

Additional differences are found in codon-choice pattern between the mycoreoviruses, even among the four related viruses. As shown in Table 4, MYRV-1/Cp9B21 has a lower frequency of [XYG + XYC] than coltiviruses or MYRV-3/RnW370 when compared between corresponding segments. It was reported previously that two phytoreoviruses, *Rice dwarf virus* (RDV) and *Wound tumor virus* (WTV), differ in codon-usage bias. That is, RDV has a higher frequency of [XYG + XYC] than WTV (Suzuki *et al.*, 1990; Suzuki, 1995). The same authors speculated that the two viruses might have adjusted their codon frequencies to their host nuclear environments, as chloroplast genes that are transferred to nuclei change their codon-frequency patterns to reflect those of nuclei more closely (Oliver *et al.*, 1990). Support for this speculation comes from the fact that monocot plants, hosts of RDV, have much higher frequency of [XYG + XYC] than dicot plants, hosts of WTV, e.g. 60·81 % for rice, 68·4 % for barley and 60·0 % for wheat vs 36·4 % for alfalfa, 39·2 % for tobacco and 37·8 % for potato. The possible role of the vector in codon-choice patterns among reoviruses has not been examined.

MYRV-1/Cp9B21, MYRV-3/RnW370 and coltiviruses infect different hosts with different codon-frequency patterns. Mice and humans, which are hosts of coltiviruses, have similar frequencies of [XYG + XYC] (59·14 and 58·93 %, respectively), whereas *C. parasitica* and *R. necatrix* have different frequencies of [XYG + XYC] (49·2 and 66·3 %, respectively) (Table 4). A large number of coding sequences of *Mus musculus*, *Homo sapiens* and *C. parasitica* are deposited in publicly available databases (http://www.kazusa.or.jp/codon/). However, because only five coding sequences are available for *R. necatrix*, it is too early to discuss the codon selection of *R. necatrix* nuclei. Mammals show greater frequencies of [XYG + XYC] than *C. parasitica*, which is consistent with the codon-selection pattern that is exhibited by coltiviruses and MYRV-1/Cp9B21. Therefore, it is tempting to speculate that codon choice in these reoviruses may have adapted to parallel that of their host nuclei more closely during the course of evolution.

The likely myristoylation of MYRV-1/Cp9B21 VP4 leads to interesting speculation about its function. One of the most thoroughly studied reovirus proteins that is known to be myristoylated is the precursor of the major outer capsid protein, μ1, which is encoded by segment M2 of mammalian orthoreovirus 3 (MRV-3) (Nibert *et al.*, 1991). Myristoylation of the complete μ1 protein at its N-terminal glycine residue promotes membrane binding of virions and entry into host cells. The myristoylated N terminus is cleaved in a reaction that is thought to be autoproteolytic, mediated by the other outer capsid protein, σ3, between N^{42} and P^{43} (Tillotson & Shatkin, 1992; Liemann *et al.*, 2002; Chandran *et al.*, 2003; Odegard *et al.*, 2004). Little is known about the requirements for proteolytic cleavage at the N terminus of μ1, but the N and P residues have been shown to be critical (Tillotson & Shatkin, 1992; Odegard *et al.*, 2004). Although nothing is known about VP4 of MYRV-1/Cp9B21 and its coltivirus homologues, the similarities between these proteins and orthoreovirus μ1 are notable: in addition to the likely N-terminal myristoylation, MYRV-1/Cp9B21, MYRV-3/RnW370 and the coltiviruses all contain an NP dipeptide at a similar position in the N-terminal region of the protein (Fig. 4a). However, we were unable to conclude that the μ1 and VP4 proteins were homologous, based on alignments of the remaining C-terminal portions of these deduced proteins. The entire VP4 sequence of MYRV-3/RnW370 aligns well with the MYRV-1/Cp9B21 sequence, so it is curious that no myristoylation site was identified on MYRV-3/RnW370. Cursory examination of its nucleotide sequence did not reveal a simple sequencing error that might be responsible for incorrect deduction of the N terminus of the putative MYRV-3/RnW370 VP4 protein. It will be interesting to determine whether this represents a real distinction between these proteins.

Similarities in amino acid sequences were noted previously among the three largest segments of four reoviruses: S1–S3 of MYRV-1/Cp9B21, MYRV-3/RnW370 and two coltiviruses (Wei *et al.*, 2003, 2004; Hillman *et al.*, 2004). The current study extends amino acid sequence similarities to other smaller segments, including S4, S5 and S6, and confirms the relatedness among the four viruses. MYRV-1/Cp9B21 is related more closely to MYRV-3/RnW370 than to coltiviruses, as judged by optimal scores and amino acid sequence identities between corresponding segments (Table 2) and phylogenetic analysis. A phylogenetic tree based on the alignment of the RdRp sequences of reoviruses (including mycoreoviruses) showed that MYRV-1/Cp9B21 and MYRV-3/RnW370 diverged from a common ancestor (Hillman *et al.*, 2004; Wei *et al.*, 2004). Similar phylogenetic relationships among mycoreoviruses, coltiviruses and fijiviruses can be seen in trees constructed from the alignments of VP2 homologues or VP6 counterparts of MYRV-1/Cp9B21 (data not shown).

Completion of the sequence determination of MYRV-1/Cp9B21 will contribute to progress on gene-product assignment and functional analyses of the encoded viral proteins. Database searches for sequence similarities and motifs have supplied clues for the elucidation of locations of viral proteins (structural or non-structural proteins). For instance, MYRV-1/Cp9B21 VP1 is very likely to be the gene for a core protein, based on the fact that all reoviral RdRp proteins examined to date are constituents of inner capsids (Mertens

et al., 2000). MYRV-1/Cp9B21 VP6 appears to be a core protein, given that the localization of its presumed homologue, NLRV P7, has been identified (Nakashima *et al.*, 1996). The similarity of MYRV-1/Cp9B21 VP3 to VP3 of several cypoviruses is intriguing, in that this protein has been demonstrated to be an outer component of BmCPV1 (Ikeda *et al.*, 2001). It will be interesting to examine the location and role of this protein in contrast to that of the putatively myristoylated VP4 protein discussed above. As demonstrated for other reoviruses (Estes, 2001; Roy, 2001), the baculovirus expression system may be useful to identify interactions among viral structural proteins.

The host fungus, *C. parasitica*, provides an excellent system to explore viral–host interactions. Robust transformation protocols are available for this host fungus (Churchill *et al.*, 1990) and efficient transfection is available for the *C. parasitica*/MYRV-1/Cp9B21 system (Hillman *et al.*, 2004). Transformation studies with genome segments alone or in combination may help with assignment of their functional roles, as has been done for the hypovirus CHV1-EP713 (Dawe & Nuss, 2001; Hillman & Suzuki, 2004). Although virulence of *C. parasitica* is greatly attenuated by infection with MYRV-1/Cp9B21, pigmentation and conidiation are slightly enhanced or not affected, relative to uninfected colonies. This phenotype is dramatically different from that of colonies infected with the well-characterized hypovirus CHV1-EP713, which suppresses virulence, conidiation and pigmentation. Recently established microarray analysis with 2200 *C. parasitica* genes (Allen *et al.*, 2003; Dawe *et al.*, 2003; Allen & Nuss, 2004) may be helpful in determining host genes that are associated specifically with hypovirulence.

ACKNOWLEDGEMENTS

The authors are grateful to C. Suzuki for analysing codon usage and B. Glasheen for technical assistance, and to Drs Max Nibert and Satoko Kanematsu for helpful discussion and access to the Odegard *et al.* (2004) and Kanematsu *et al.* (2004) papers before publication.

REFERENCES

Allen, T. D. & Nuss, D. L. (2004). Specific and common alterations in host gene transcript accumulation following infection of the chestnut blight fungus by mild and severe hypoviruses. *J Virol* **78**, 4145–4155.

Allen, T. D., Dawe, A. L. & Nuss, D. L. (2003). Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypoviruses. *Eukaryot Cell* **2**, 1253–1265.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Asamizu, T., Summers, D., Motika, M. B., Anzola, J. V. & Nuss, D. L. (1985). Molecular cloning and characterization of the genome of wound tumor virus: a tumor-inducing plant reovirus. *Virology* **144**, 398–409.

Attoui, H., Mohd Jaafar, F., Biagini, P., Cantaloube, J.-F., de Micco, P., Murphy, F. A. & de Lamballerie, X. (2002). Genus *Coltivirus* (family *Reoviridae*): genomic and morphologic characterization of Old World and New World viruses. *Arch Virol* **147**, 533–561.

Bologna, G., Yvon, C., Duvaud, S. & Veuthey, A.-L. (2004). *N*-Terminal myristoylation predictions by ensembles of neural networks. *Proteomics* **4**, 1626–1632.

Chandran, K., Parker, J. S. L., Ehrlich, M., Kirchhausen, T. & Nibert, M. L. (2003). The δ region of outer-capsid protein μ1 undergoes conformational change and release from reovirus particles during cell entry. *J Virol* **77**, 13361–13375.

Churchill, A. C. L., Ciuffetti, L. M., Hansen, D. R., Van Etten, H. D. & Van Alfen, N. K. (1990). Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. *Curr Genet* **17**, 25–31.

Dawe, A. L. & Nuss, D. L. (2001). Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. *Annu Rev Genet* **35**, 1–29.

Dawe, A. L., McMains, V. C., Panglao, M., Kasahara, S., Chen, B. & Nuss, D. L. (2003). An ordered collection of expressed sequences from *Cryphonectria parasitica* and evidence of genomic microsynteny with *Neurospora crassa* and *Magnaporthe grisea*. *Microbiology* **149**, 2373–2384.

Enebak, S. A. (1992). *Characterization of dsRNA-containing strains of Cryphonectria parasitica recovered from the central Appalachian.* PhD thesis, West Virginia University, WV, USA.

Enebak, S. A., Hillman, B. I. & MacDonald, W. L. (1994). A hypovirulent isolate of *Cryphonectria parasitica* with multiple, genetically unique dsRNA segments. *Mol Plant Microbe Interact* **7**, 590–595.

Estes, M. K. (2001). Rotaviruses and their replication. In *Fields Virology*, 4th edn, vol. 2, pp. 1747–1785. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Grimes, J. M., Burroughs, J. N., Gouet, P., Diprose, J. M., Malby, R., Ziéntara, S., Mertens, P. P. C. & Stuart, D. I. (1998). The atomic structure of the bluetongue virus core. *Nature* **395**, 470–478.

Hillman, B. I. & Suzuki, N. (2004). Viruses in the chestnut blight fungus. *Adv Virus Res* (in press).

Hillman, B. I., Supyani, S., Kondo, H. & Suzuki, N. (2004). A reovirus of the fungus *Cryphonectria parasitica* that is infectious as particles and related to the *Coltivirus* genus of animal pathogens. *J Virol* **78**, 892–898.

Ikeda, K., Nagaoka, S., Winkler, S., Kotani, K., Yagi, H., Nakanishi, K., Miyajima, S., Kobayashi, J. & Mori, H. (2001). Molecular characterization of *Bombyx mori* cytoplasmic polyhedrosis virus genome segment 4. *J Virol* **75**, 988–995.

Isogai, M., Uyeda, I. & Lee, B. (1998). Detection and assignment of proteins encoded by rice black streaked dwarf fijivirus S7, S8, S9 and S10. *J Gen Virol* **79**, 1487–1494.

Kanematsu, S., Arakawa, M., Oikawa, Y. & 10 other authors (2004). A reovirus causes hypovirulence of *Rosellinia necatrix*. *Phytopathology* **94**, 561–568.

Kudo, H., Uyeda, I. & Shikata, E. (1991). Viruses in the Phytoreovirus genus of the *Reoviridae* family have the same conserved terminal sequences. *J Gen Virol* **72**, 2857–2866.

Lambden, P. R., Cooke, S. J., Caul, E. O. & Clarke, I. N. (1992). Cloning of noncultivatable human rotavirus by single primer amplification. *J Virol* **66**, 1817–1822.

Lawton, J. A., Estes, M. K. & Prasad, B. V. V. (1997a). Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat Struct Biol* **4**, 118–121.

Lawton, J. A., Zeng, C. Q.-Y., Mukherjee, S. K., Cohen, J., Estes, M. K. & Prasad, B. V. V. (1997b). Three-dimensional structural

analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: implications for the architecture of the VP2 capsid layer. *J Virol* **71**, 7353–7360.

Liemann, S., Chandran, K., Baker, T. S., Nibert, M. L. & Harrison, S. C. (2002). Structure of the reovirus membrane-penetration protein, μ1, in a complex with its protector protein, σ3. *Cell* **108**, 283–295.

Lipman, D. J. & Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.

Maruyama, K. & Sugano, S. (1994). Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* **138**, 171–174.

Maurer-Stroh, S., Gouda, M., Novatchkova, M., Schleiffer, A., Schneider, G., Sirota, F. L., Wildpaner, M., Hayashi, N. & Eisenhaber, F. (2004). MYRbase: analysis of genome-wide glycine myristoylation enlarges the functional spectrum of eukaryotic myristoylated proteins. *Genome Biol* **5**, R21.

McQualter, R. B., Burns, P., Smith, G. R., Dale, J. L. & Harding, R. M. (2003). Molecular analysis of *Fiji disease virus* genome segments 5, 6, 8 and 10. *Arch Virol* **149**, 713–721.

Mertens, P. P. C., Arella, M., Attoui, H. & 41 other authors (2000). Family *Reoviridae*. In *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 395–480. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.

Mertens, P. P. C., Hillman, B. I. & Suzuki, N. (2004). Genus *Mycoreovirus*. In *Virus Taxonomy: Eighth Report of the International Committee for the Taxonomy of Viruses*. Edited by C. M. Fauquet *et al.* San Diego: Academic Press (in press).

Nakagawa, A., Miyazaki, N., Taka, J. & 9 other authors (2003). The atomic structure of *Rice dwarf* virus reveals the self-assembly mechanism of component proteins. *Structure (Camb)* **11**, 1227–1238.

Nakashima, N., Koizumi, M., Watanabe, H. & Noda, H. (1996). Complete nucleotide sequence of the *Nilaparvata lugens* reovirus: a putative member of the genus *Fijivirus*. *J Gen Virol* **77**, 139–146.

Nibert, M. L. & Schiff, L. A. (2001). Reoviruses and their replication. In *Fields Virology*, 4th edn, vol. 2, pp. 1679–1729. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Nibert, M. L. & Kim, J. (2004). Conserved sequence motifs for nucleoside triphosphate binding unique to turreted *Reoviridae* members and coltiviruses. *J Virol* **78**, 5528–5530.

Nibert, M. L., Schiff, L. A. & Fields, B. N. (1991). Mammalian reoviruses contain a myristoylated structural protein. *J Virol* **65**, 1960–1967.

Odegard, A. L., Chandran, K., Zhang, X., Parker, J. S. L., Baker, T. S. & Nibert, M. L. (2004). Putative autocleavage of outer capsid protein μ1, allowing release of myristoylated peptide μ1N during particle uncoating, is critical for cell entry by reovirus. *J Virol* **78**, 8732–8745.

Oliver, J. L., Marín, A. & Martínez-Zapater, J. M. (1990). Chloroplast genes transferred to the nuclear plant genome have adjusted to nuclear base composition and codon usage. *Nucleic Acids Res* **18**, 65–73.

Osaki, H., Wei, C. Z., Arakawa, M., Iwanami, T., Nomura, K., Matsumoto, N. & Ohtsu, Y. (2002). Nucleotide sequences of double-stranded RNA segments from a hypovirulent strain of the white root rot fungus *Rosellinia necatrix*: possibly of the first member of the *Reoviridae* from fungus. *Virus Genes* **25**, 101–107.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.

Polashock, J. J. & Hillman, B. I. (1994). A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. *Proc Natl Acad Sci U S A* **91**, 8680–8684.

Rao, S., Carner, G. R., Scott, S. W., Omura, T. & Hagiwara, K. (2003). Comparison of the amino acid sequences of RNA-dependent RNA polymerases of cypoviruses in the family *Reoviridae*. *Arch Virol* **148**, 209–219.

Reinisch, K. M., Nibert, M. L. & Harrison, S. C. (2000). Structure of the reovirus core at 3·6 Å resolution. *Nature* **404**, 960–967.

Roy, P. (2001). Orbiviruses. In *Fields Virology*, 4th edn, vol. 2, pp. 1835–1869. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Stuart, D. I., Gouet, P., Grimes, J., Malby, R., Diprose, J., Ziéntara, S., Burroughs, J. N. & Mertens, P. P. C. (1998). Structural studies of orbivirus particles. *Arch Virol Suppl* **14**, 235–250.

Suzuki, N. (1995). Molecular analysis of the rice dwarf virus genome. *Semin Virol* **6**, 89–95.

Suzuki, N., Watanabe, Y., Kusano, T. & Kitagawa, Y. (1990). Sequence analysis of rice dwarf phytoreovirus genome segments S4, S5, and S6: comparison with the equivalent wound tumor virus segments. *Virology* **179**, 446–454.

Suzuki, N., Maruyama, K., Moriyama, M. & Nuss, D. L. (2003). Hypovirus papain-like protease p29 functions in *trans* to enhance viral double-stranded RNA accumulation and vertical transmission. *J Virol* **77**, 11697–11707.

Tao, Y., Farsetta, D. L., Nibert, M. L. & Harrison, S. C. (2002). RNA synthesis in a cage – structural studies of reovirus polymerase λ3. *Cell* **111**, 733–745.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Tillotson, L. & Shatkin, A. J. (1992). Reovirus polypeptide σ3 and N-terminal myristoylation of polypeptide μ1 are required for site-specific cleavage to μ1C in transfected cells. *J Virol* **66**, 2180–2186.

Wei, C. Z., Osaki, H., Iwanami, T., Matsumoto, N. & Ohtsu, Y. (2003). Molecular characterization of dsRNA segments 2 and 5 and electron microscopy of a novel reovirus from a hypovirulent isolate, W370, of the plant pathogen *Rosellinia necatrix*. *J Gen Virol* **84**, 2431–2437.

Wei, C. Z., Osaki, H., Iwanami, T., Matsumoto, N. & Ohtsu, Y. (2004). Complete nucleotide sequences of genome segments 1 and 3 of Rosellinia anti-rot virus in the family *Reoviridae*. *Arch Virol* **149**, 773–777.

Xu, Z., Anzola, J. V., Nalin, C. M. & Nuss, D. L. (1989). The 3′-terminal sequence of a wound tumor virus transcript can influence conformational and functional properties associated with the 5′-terminus. *Virology* **170**, 511–522.