Mycoreovirus 1 S4-coded protein is dispensable for viral replication but necessary for efficient vertical transmission and normal symptom induction

Ana Eusebio-Cope, Liying Sun, Bradley I. Hillman, Nobuhiro Suzuki

Abstract

Rearrangements of two segments, S6 and S10, of Mycoreovirus 1 (MyRV1), a member of the family Reoviridae, were previously shown to be induced at a high rate by the multifunctional protein p29 encoded by a distinct ssRNA virus, the prototype hypovirus CHV1-EP713 (Sun and Suzuki, RNA 14, 2557–2571, 2008). Here we report the occurrence of rearrangements of MyRV1 S4, albeit at a very low frequency, in the absence of CHV1 p29, resulting in internal 80–90% deletions of the open reading frame (ORF) in S4. Comparative analyses of fungal strains infected by wild-type MyRV1 and its variants carrying rearrangements of S4, S4 plus S10 and S10 indicated that S4-encoded VP4, like VP10, is non-essential for virus replication but required for efficient vertical transmission and symptom expression caused by MyRV1. This is the first example of a reovirus variant that carries deletions of over 75% of the ORFs in two genome segments and is still replication-competent.

Introduction

The family Reoviridae, one of the largest virus families, includes a variety of members infecting fungi, plants, insects, fish, and higher vertebrates including humans (Mertens et al., 2005a). Genomes composed of 9 to 12 segmented dsRNA are enclosed in multilayered particles, with virus core particles performing all the enzymatic activities responsible for the synthesis of virus mRNAs. The genus Mycoreovirus, relatively newly established within the family, contains three members: Mycoreovirus 1 (MyRV1), MyRV2, and MyRV3 (Mertens et al., 2005b). The first two were isolated from the chestnut blight fungus, Cryphonectria parasitica, and have 11 dsRNA genome segments (S1 to S11, ranging from 4127 to 732 in bp for MyRV1) (Enebak et al., 1994; Hillman and Suzuki, 2004; Suzuki et al., 2004). MyRV3, with a genome of 12 dsRNA segments, isolated from another phytopathogenic filamentous fungus, Rosellinia necatrix, shows moderate levels of sequence similarity to MyRV1 and MyRV2 (Hillman et al., 2004; Suzuki et al., 2004; Wei et al., 2004). Significant sequence similarities are also found among some equivalent genome segments of the three viruses and mammal-infecting members of the genus Coltivirus (Hillman et al., 2004; Suzuki et al., 2004; B.I. Hillman, unpublished results), suggesting that the coltiviruses are the closest known relatives of the mycoreoviruses. Biochemical and bioinformatics analyses have shown that MyRV1 S1-, S3-, S4-, and S6-coded proteins (VP1, VP3, VP4, VP6) have domains for RNA-dependent RNA polymerase (RdRp), guanylyltransferase, and NTP-binding, respectively, while the S4 product (VP4) is predicted to be a target for N-myristoylation by a cellular myristoyltransferase (Hillman et al., 2004; Supyani et al., 2007; Suzuki et al., 2004).

Rearrangements of individual genome segments, including extensions and/or deletions of ORFs, are common to members of the Reoviridae (Desselberger, 1996; Nuss, 1984; Taniguchi and Urasawa 1995). In most cases, the terminal sequence domains, believed to be essential for RNA replication and segment sorting for packaging, are retained in otherwise rearranged segments (Anzola et al., 1987). Mechanisms mediated by RdRp and template structures including direct and inverted repeats are suggested for the events (Kojima et al., 1996; Gault et al., 2001; Matthijssens et al., 2006), and such genome rearrangements have contributed to functional analyses of members of the family Reoviridae. Genome rearrangements have contributed to functional analyses of genome segments of members of the family....
Reoviridae. For example Wound tumor virus segments S2 and S5 were found to be dispensable for replication and maintenance in the absence of the insect vector (Nuss, 1984), and reassortant studies with rotaviruses have been used to map numerous phenotypic mutants (see Ramig, 1997 for review).

Two mechanistically distinct mutation events were recently reported for members of the genus Mycoreovirus. One of the MyRV3 genome segments, S8, was completely lost during subculturing of fungal colonies originally infected with the wild-type virus, resulting in no substantial reduction of the replication competency (Kanematsu et al., 2004). To our knowledge, this is the only example in which a reovirus segment was completely lost and no cognate remnant RNA was found following a rearrangement event.

In studies of interactions between MyRV1 and the prototype hypovirus CHV1-EP713, which has an undivided single-stranded RNA genome in the RdRp supergroup 1 (picorna-like) lineage, or its transgenically expressed multifunctional protein p29, mutation of the reovirus genomic segments were also found to occur at a high frequency (Sun and Suzuki, 2008). Substantial rearrangement of two MyRV1 segments were reported: in one instance, deletion of S10 resulted in two size variants, S10s1 and S10s2 that lack approximately 74.2% and 76.4% of the coding domain of intact S10; in the other case, the coding sequence of S6 was almost entirely duplicated resulting in a segment S6L with an in-frame duplication (97%) of authentic S6. The MyRV1 rearranged segments retained their terminal sequences. Biological characterization of MyRV1 variants with the S10 and S6 rearranged segments showed that S10-coded VP10, albeit dispensable for virus viability, is involved in repression of the growth of aerial hyphae and virulence, while the longer version of VP6 was not associated with discernable alterations to the phenotype caused by wild-type MyRV1.

While screening a collection of C. parasitica transformants for host factors associated with MyRV1 symptom induction using an approach similar to that developed for the CHV1/C. parasitica system (Faruk et al., 2008a, 2008b), we found a very low frequency of CHV1 p29-independent rearrangements of MyRV1 S4. MyRV1 S4 underwent internal deletion of 78–89% of the coding region, resulting in the emergence of shorter variants of S4 (S4ss). A reassortant carrying deletions in segments 4 and 10, with neither complete segment, was generated by anastomosing MyRV1/S4ss with MyRV1/S10ss-infected fungal colonies. Comparative analyses of fungal strains infected by MyRV1/S4ss, MyRV1/S10ss, and MyRV1/S4ss + S10ss revealed that, like VP10, S4-encoded VP4 is non-essential for virus replication, but is required for efficient vertical transmission and symptom expression caused by MyRV1. This study provides an additional example of MyRV1 rearrangement that will contribute to functional analysis of its genome.

### Results

**Rearrangement of MyRV1 segment S4 in fungal transformants with a mutagenic plasmid**

We developed a screening method for host factors associated with symptom induction and replication of CHV1 (Faruk et al., 2008a, 2008b). Using a similar approach, we transformed spheroplasts derived from MyRV1-infected EP155 with the mutagenic plasmid pHygR. A collection of transformed fungal isolates were screened initially to identify host factors associated with replication and symptom induction of MyRV1. Through visual inspection of a total of 1900 fungal colonies, we pre-selected 12 transformants showing unusual phenotypes for MyRV1 symptoms. We found that four of the 12 fungal isolates showed phenotypic fluctuation during storage and subculturing. SDS-polyacrylamide gel electrophoretic analysis of dsRNA from the two transformants with at least three copies of pHygR, termed Tphph3c and Tphph17 (Table 1, Supplementary Fig. 1), showed unusual electrophoretotypes: less intensity of S4 bands and emergence of smaller segments, presumed to have internal deletions, at the site of infected fungal colonies originally infected with the wild-type virus, resulting in no substantial reduction of the replication competency (Kanematsu et al., 2004). To our knowledge, this is the only example in which a reovirus segment was completely lost and no cognate remnant RNA was found following a rearrangement event.

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**Table 1**  

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Viral</strong></td>
<td></td>
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<tr>
<td>MyRV1-Cp9B21</td>
<td>Prototype of the genus Mycoreovirus</td>
<td>Hillman et al., 2004</td>
</tr>
<tr>
<td>MyRV1/S4ss1</td>
<td>Variant with rearranged S4ss1 lacking 88.5% of the S4 coding domain</td>
<td>This study</td>
</tr>
<tr>
<td>MyRV1/S4ss2</td>
<td>Variant with rearranged S4ss2 lacking 83.6% of the S4 coding domain</td>
<td>This study</td>
</tr>
<tr>
<td>MyRV1/S4ss3</td>
<td>Variant with rearranged S4ss2 and S4ss3 lacking 79.2% of the S4 coding domain</td>
<td>This study</td>
</tr>
<tr>
<td>MyRV1/S4ss4</td>
<td>Variant with rearranged S4ss4 lacking 78.4% of the S4 coding domain</td>
<td>This study</td>
</tr>
<tr>
<td>MyRV1/S10ss2</td>
<td>CHV1 p29-induced mutant with rearranged S10ss2 lacking 76.4% of the S10 ORF</td>
<td>Sun and Suzuki, 2008</td>
</tr>
<tr>
<td>MyRV1/S4ss1 + S10ss2</td>
<td>Variant with rearranged S4ss1 and S10ss2</td>
<td>This study</td>
</tr>
<tr>
<td>MyRV1/S4ss4 + S10ss2</td>
<td>Variant with rearranged S4ss4 and S10ss2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Fungal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP155</td>
<td>Virus-free field isolate</td>
<td>ATCC 38755</td>
</tr>
<tr>
<td>Tphph3c</td>
<td>EP155 transformant carrying at least three copies of the mutagenic plasmid pHygR</td>
<td>This study</td>
</tr>
<tr>
<td>Tphph17</td>
<td>EP155 transformant carrying at least three copies of the mutagenic plasmid pHygR</td>
<td>This study</td>
</tr>
<tr>
<td>Twp29-32F</td>
<td>Transformant of EP155 with the p29 coding sequence</td>
<td>Sun et al., 2006</td>
</tr>
</tbody>
</table>
Of those segments, S10 underwent rearrangement most frequently. As shown in Fig. 1, authentic MyRV1 S10 was retained in an intact form in the S4ss-bearing strains. Similarly, probing these blots with the other 9 segments of MyVR1 demonstrated no alterations in segments other than S4 and S10 (data not shown), suggesting that all segments but S4 remained intact in these mutants.

To determine genetic organization of the shorter versions of S4, RT-PCR was carried out using gel-purified dsRNA fragments as template and a primer set flanking the segment termini (Supyani et al., 2007). As shown in Fig. 2A, DNA fragments of 2.3 kb were amplified on authentic S4 coding for VP4 of 720 amino acids (aa) (Fig. 2B), whereas fragments of smaller sizes corresponding to the sizes of the S4ss variants were generated for the MyRV1/S4ss mutants (Fig. 2A, lanes 2 to 5). For example, MyRV1/S4ss1 provided DNA fragments of approximately 0.3 kb, while MyRV1/S4ss4 resulted in DNA fragments of approximately 0.7 kb. At least 5 clones were sequenced for each fragment, and terminal sequences were confirmed by 5’-RACE.

Schematic representations of the S4ss segments are shown in Fig. 2B. S4ss1 to S4ss4 appear to have arisen from simple internal deletion events, resulting in loss of 78.4% (S4ss4) to 88.5% (S4ss1) of the authentic ORF. The four S4ss variants differed from one another in deletion endpoint at both 5’ and 3’ regions. In each case, the N and C terminal portions of S4ss1, S4ss3 and S4ss4 were linked in-frame, encoding deduced proteins of 82 aa, 149 aa, and 155 aa, respectively. Rearrangements found in S4ss2 resulted in an out-of-frame ORF connection of the terminal regions: a small ORF that would code for 62 aa identical to the N-terminal portion of VP4 was linked to 7 aa unrelated to VP4.

We previously found that S10ss carried mismatch mutations at or close to the deletion junctions (Sun and Suzuki, 2008), as reported by Lai (1992) for aberrant homologous recombination. Mismatches were observed at the 5’ terminal deletion points of S4ss2, but not in the other S4ss variants.

Minimal effect of internal deletion of S4 on virus accumulation

It was anticipated that the deletion of large portions of VP4 (Fig. 2B) might impair the replication of MyRV1. To explore this possibility we compared virus accumulation using two methods. Total RNA fractions isolated from EP155 infected with the wild-type and S4-variants of MyRV1 (MyRV1/S4ss1, MyRV1/S4ss2, MyRV1/S4ss3, and MyRV1/S4ss4) were analyzed by agarose gel electrophoresis (Fig. 3A). In comparison with wild-type MyRV1, no significant decrease in genomic RNA accumulation was observed for MyRV1/S4ss1; rather, a slight increase was seen for the other three variants when evaluated from the band intensity of S1+S2 or S3. It was noteworthy that S4 was detectable in the wild-type strain, but not in the variants. Secondly, Northern blot analysis (Fig. 3B) was performed, and this revealed no significant decrease in genomic RNA accumulation was observed for MyRV1/S4ss1; rather, a slight increase was seen for the other three variants when evaluated from the band intensity of S1 + S2 or S3. It was noteworthy that S4 was detectable in the wild-type strain, but not in the variants. None of the probes detected a specific hybridization signal in ssRNA isolated from virus-free EP155.

Expression of S4 and S4ss

Northern blot analysis was first performed using the same set of ssRNAs as that for Fig. 3B to explore the expression of S4 and S4ss. As a result, the S4-specific probe allowed detection of transcripts from S4 and S4ss1 to S4ss4 in RNA preparations from fungal colonies infected with the respective viral strains (Fig. 4A). The migration positions of S4
and S4ss1 to S4ss4 were in agreement with the results shown in Figs. 1
and 2. Assuming the core particles to be the site of transcription, these
results suggested that the S4ss segments were packageable.

To examine whether mutants express the complete protein VP4
and its shorter version VP4ss, we prepared antiserum against
recombinant VP4 and used it to detect VP4 in MyRV1-infected
mycelia. VP4 was difficult to detect in total protein fractions obtained
from mycelia, and thus, we used proteins from spheroplasts. The
fractions obtained from MyRV1-infected spheroplasts after centrifu-
gation at 1000 × g for 10 min were probed by antibodies specific for
MyRV1 VP2, VP4 and VP9. A protein band showing the same
migration as recombinant VP4 expressed in insect cells via baculo-

Fig. 2. RT-PCR analysis and schematic organization of MyRV1 rearranged segments. (A)
Agarose gel electrophoresis of RT-PCR products on the S4 rearranged segments. S4ss1
to S4ss4 in Fig. 1A was eluted and used as template in cDNA synthesis and subsequent
PCR using a primer set S4FL1 and S4FL2 (Supyani et al., 2007). Amplified products from
authentic S4 (lane 1) and S4ss1 to S4ss4 (lanes 2 to 5) were electrophoresed in agarose
1.4% gel in 0.5× TAE buffer. Size standards were electrophoresed in parallel (lane M).
(B) Diagram of the organization of MyRV1 RNA segment S4s revealed by sequencing of
the RT-PCR fragments. MyRV1 normal S4 is 2269 bp in length, encoding 720 aa, starting
at the AUG (map positions 39–41) and ending at 2199–2201 (Suzuki et al., 2004). S4ss1
to S4ss4 originated from S4 by internal deletion events retained the 5′ terminal (nt
positions 1 to 185–277) and 3′ terminal portions (nt positions 1968–2100 to 2269). The
5′- and 3′-terminal portions were connected in an in-frame (S4ss1, S4ss3, S4ss4) or
out-of-frame fashion (S4ss2). Shaded boxes denote ORFs and numbers refer to
positions of start and stop codons, and deletion breakpoints.

Fig. 3. Agarose gel and Northern blot analyses of wild-type and variant strains of
MyRV1. (A) Agarose gel electrophoresis of total RNA fractions of EP155 infected with
the viral strains. Total RNA was isolated from EP155 infected with MyRV1, MyRV1/ S4ss1, MyRV1/S4ss2, MyRV1/S4ss3, and MyRV1/S4ss4, and applied to wells of 1.4% agarose gel. The position of authentic S4 is shown by the arrow. Migration positions of S1 to S4 indicated on the right. (B) Northern blot analysis of wild-type and variant
MyRV1 strains. Single-stranded RNA fractions were obtained from fungal colonies
infected with wild-type MyRV1, and strains carrying rearranged segments (MyRV1/
S4ss1, MyRV1/S4ss2, MyRV1/S4ss3, and MyRV1/S4ss4). The RNA fractions were
electrophoresed under denaturing conditions. After being transferred to nylon
membrane, RNA was probed with DIG-labeled PCR products specific for S1, S2, and
S10. Ribosomal RNAs (rRNA) stained by EtBr were used as loading markers.

viruses vectors (Fig. 4B/αVP4, lane VP4) was observed in spheroplasts
infected with wild-type MyRV1 (Fig. 4B, lane MyRV1). This band was
not observed in the fractions from virus-free EP155 (lane EP155) or
fungal strains infected with the MyRV1 variants carrying S4ss (lanes
MyRV1/S4ss1, MyRV1/S4ss2, MyRV1/S4ss3, and MyRV1/S4ss4). However, accumulation
of a possible major capsid protein VP2 (Fig. 4B/αVP2) and a
possible non-structural protein VP9 (Fig. 4/αVP9) was confirmed in
all the virus-infected fungal strains. A polypeptide serologically
related to VP9 but with greater mobility than authentic VP9 (shown
by the asterisks) was detected in fungal strains infected with the
MyRV1 variants, but the specific nature of this apparently smaller
protein remains unknown. In each of the variants, the smaller protein
was observed at approximately the same level as the authentic VP9.
We used a 20% polyacrylamide gel for detection of VP4ss, considering
their expected sizes of VP4ss1 to VPss4 that would range from 8 kDa
to 17 kDa. Consequently we failed to detect possible VP4ss in fungal
strains infected with any of the variant viruses (data not shown), as in
the case of VP10ss (Sun and Suzuki, 2008).
Northern and Western blot analyses of the wild-type and variant strains of MyRV1. (A) Detection of MyRV1 S4 transcripts by Northern blotting. The same RNA preparations as used in Fig. 3B were examined by the same method except that a MyRV1 S4-specific probe was used. (B) Detection of MyRV1 S4-encoded proteins. Proteins in the pellet fractions obtained by centrifugation at 1000 × g for 10 min of spheroplast lysates of fungal culture infected wild-type and S4ss-containing variants were resolved in an SDS-polyacrylamide gel (7.5%), and transferred to cellulose membrane. The resultant membrane were stripped horizontally into three pieces containing proteins with certain ranges of molecular masses, and each sheet was probed by an anti-MyRV1 VP2, VP4, or VP9 antisera (shown on the right). Proteins in spheroplasts from virus-free EP155 and insect cells infected by baculovirus vectors carrying MyRV1 S2, S4 and S9 full-length cDNAs cells (Supyani et al., 2007) were analyzed in parallel. Migration positions of molecular mass standards (150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, and 25 kDa) (Fermentas) are shown on the left.

**Effects of S4 rearrangements on vertical transmission**

To examine the effects of S4 rearrangements on virus transmission, single-conidial isolates were tested for virus infection by visual estimation and for subsequent genome segment composition by Northern blotting. Intriguingly, frequency of vertical transmission of S4ss-containing MyRV1 variants was much lower than the 8.8% transmission rate of wild-type MyRV1 (Table 2), consistent with the previous report by Sun and Suzuki (2008). In the present study, S4-encoded VP4 was found independently to be dispensable for MyRV1 replication (Fig. 3A). Thus, it was of great interest to determine whether a strain of MyRV1 containing deletions in both S4 and S10 could be viable. To prepare fungal isolates infected with MyRV1/S4ss+S10ss, virus-free EP155 was fused simultaneously with MyRV1/S4ss1-infected and MyRV1/S10ss2-infected colonies. After receiving both the viral strains, plugs of mycelia from the EP155 side were subcultured. The resulting cultures contained authentic (S4 and S10) and rearranged (S4ss and S10ss) segments. To increase the ratio in amounts of rearranged segments,

**Table 2**

<table>
<thead>
<tr>
<th>Virus strain detected in the next generation</th>
<th>EP155 infected with</th>
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<tr>
<td>MyRV1</td>
<td>MyRV1/S4ss1</td>
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<tr>
<td>MyRV1</td>
<td>35</td>
</tr>
<tr>
<td>MyRV1/S4ss</td>
<td>361</td>
</tr>
<tr>
<td>MyRV1/S4ss+S10ss</td>
<td>396</td>
</tr>
<tr>
<td>MyRV1/S10ss</td>
<td>8.8</td>
</tr>
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</table>

Single conidial isolates originated from fungal colonies infected with the wild-type or variant MyRV1 were tested for presence of virus.

Morphological properties associated with S4ss

As previously reported by Hillman et al. (2004), EP155 infected with wild-type MyRV1 grew slightly slower and was reduced in growth of aerial hyphae relative to EP155, while producing deep orange-brown pigments. Colonies infected with the S4ss-containing variants were readily differentiated from those infected with the wild-type MyRV1. As shown in Fig. 5A, colonies infected with any of the S4ss-carrying variants had irregular margins with submerged lobed-shaped, whitish edges and rarely reached to the edge of a culture dish. The central regions of fungal colonies infected with MyRV1/S4ss were darker than those infected with the wild-type virus.

Virus-free EP155 induced large lesions of nearly 12 cm² in virulence assays on apple fruits, while EP155 infected with wild-type MyRV1 induced extremely small lesions (approximately 1 cm²) (Fig. 5B), thus being regarded as a virulence attenuation agent potential (Ghabrial and Suzuki, 2009). Despite the differences in colony morphology (Fig. 5A), EP155 infected with MyRV1/S4ss1 to MyRV1/S4ss4 induced lesions similar in size to those induced by wild-type MyRV1.

Consistent with our previous report (Hillman et al., 2004), MyRV1-infected colonies sporulated to a level comparable to virus-free EP155 (∼10⁸ spores/ml vs. ∼10⁶ spores/ml). Sporulation levels of fungal colonies infected with the S4ss-harboring strains were comparable to those of fungal colonies infected with the wild-type virus (Fig. 5B).

These combined results suggested that S4 contributed to phenotypic alterations, but not to virulence attenuation, caused by MyRV1.

**Generation and characterization of MyRV1/S4ss1 + S10ss2, MyRV1/S4ss4 + S10ss2**

We had previously reported that a MyRV1 variant with S10ss lacking three fourths of the coding region was still replication-competent and induced symptoms different from those of wild-type MyRV1 (Sun and Suzuki, 2008). In the present study, S4-encoded VP4 was found independently to be dispensable for MyRV1 replication (Fig. 3A). Thus, it was of great interest to determine whether a strain of MyRV1 containing deletions in both S4 and S10 could be viable. To prepare fungal isolates infected with MyRV1/S4sS+S10ss, virus-free EP155 was fused simultaneously with MyRV1/S4ss1-infected and MyRV1/S10ss2-infected colonies. After receiving both the viral strains, plugs of mycelia from the EP155 side were subcultured. The resulting cultures contained authentic (S4 and S10) and rearranged (S4ss and S10ss) segments. To increase the ratio in amounts of rearranged segments,
those colonies were continuously subcultured for 2 months, and then allowed to sporulate for single spore isolation. We screened a number of single-conidial isolates for reasortants harboring the S4 and S10 rearrangements. We first analyzed them for virus infection by visual inspection of their phenotype, and then tested the electropherotype of their dsRNA by SDS-PAGE.

Of 565 single-conidial isolates tested only 11 were found virus-infected, and of the 11, 8 contained both S4.ss1 and S10.ss2. Similarly a double mutant carrying S4.ss4 + S10.ss2 was isolated. To eliminate the possibility of mixed infection by virus strains carrying a minor amount of authentic S4 or S10, we repeated two rounds of single spore isolation procedures with the newly infected colonies.

In SDS-PAGE analysis of the genomic RNA of two resultant isolates (MyRV1/S4.ss1+S10.ss2, MyRV1/S4.ss4+S10.ss2) [Fig. 6A] and confirmatory Northern analysis (Fig. 6B), the absence of normal S4 and S10 and the presence of S4.ss and S10.ss are evident. That is, the double-mutant MyRV1/S4.ss1+S10.ss2 produced shorter versions of S4 and S10 transcripts, indicating the ability of MyRV1/S4.ss1+S10.ss2 to form active transcriptase complexes and utilize S4.ss and S10.ss as their templates, while the single mutants MyRV1/S4.ss1 and MyRV1/S10.ss2 synthesized transcripts of either S4 or S10, respectively. MyRV1/S4.ss4+S10.ss2 provided a Northern profile similar to that of MyRV1/S4.ss1+S10.ss2 shown in Fig. 6B. Western analysis confirmed the absence of VP4 in spheroplasts infected with MyRV1/S4.ss1+S10.ss2 (data not shown) and MyRV1/S4.ss4+S10.ss2 (Fig. 4B). Northern analysis with S1- and S2-specific probes allowed comparison of replication levels among virus strains carrying single and double rearrangements (Fig. 6B). Surprisingly, like MyRV1/S4.ss, the mutants MyRV1/S4.ss1+S10.ss2 (Fig. 6B) and MyRV1/S4.ss4+S10.ss2 (data not shown) exhibited levels of replication comparable to that of wild-type MyRV1 or the single mutants MyRV1/S4.ss1 and MyRV1/S10.ss2. These results clearly indicate that the two viral proteins encoded by S4 (VP4) and S10 (VP10) are dispensable for virus viability.

Rates of vertical transmission through asexual spores of MyRV1/S4.ss1+S10.ss2 (1.7%) and MyRV1/S4.ss4+S10.ss2 (2.0%) were severely reduced relative to wild-type (8.8%) or MyRV1/S10.ss2 (7.0%), but were slightly increased compared to MyRV1/S4.ss variants (0.3–1.3%) (Table 2). The decrease in vertical transmission found in S4ss-containing MyRV1 variants was statistically significant (p<0.01).

**Fungal colonies infected by double-mutant MyRV1/S4.ss + S10.ss and single mutant MyRV1/S4.ss have similar morphological attributes**

As reported previously by our group (Sun and Suzuki, 2008), colonies infected with MyRV1/S10.ss2 grew more aerial mycelia than wild-type MyRV1-infected colonies. Interestingly, fungal colonies infected with MyRV1/S4.ss1 + S10.ss2 and MyRV1/S4.ss4 + S10.ss2 showed reduced growth rates and reduction in the growth of aerial hyphae with whitish colony edges, similar symptoms to those induced by MyRV1/S4.ss4 (Fig. 7A). Those symptoms were distinguishable from symptoms induced by MyRV1/S10.ss2 (Fig. 7A). Levels of condiation in fungal colonies infected by MyRV1/S4.ss1 + S10.ss2 (data not shown) and MyRV1/S4.ss4 + S10.ss2 (Fig. 7B) were comparable to those of colonies infected by MyRV1/S4.ss4 (Fig. 7B), but slightly lower than those of virus-free EP155 or wild-type MyRV1-infected colonies. The double-mutant strain reduced the virulence of the fungus as markedly as the wild-type MyRV1 and MyRV1/S4.ss4. Lesions induced on apples by MyRV1/S10.ss2 were slightly larger than those induced by MyRV1/S4.ss4 + S10.ss2 or MyRV1/S4.ss4, but much smaller than those induced by virus-free EP155, suggesting that the symptom expression associated with S4ss is dominant over that associated with MyRV1/S10.ss2 upon infection by the double mutants.

**Discussion**

Genome rearrangements are common phenomena among members of the family Reoviridae, e.g., the genera Phytoreovirus (Murao et al., 1996; Nuss, 1984), Oryzavirus (Maoka et al., 1993), Orvirus (Eaton and Gould, 1987), Cypovirus (Arella et al., 1988), Rotavirus (Desselberger, 1987; Schnepf et al., 2008; Taniguchi and Urasawa, 1995), and Orthoreovirus (Ni and Kemp, 1994). Such rearrangements occur spontaneously in infected individuals or under laboratory conditions that include serial passage in host cell cultures particularly at a high m.o.i. and continuous passage or maintenance exclusively in one of multiple hosts for vectored reoviruses. Members of the genus Mycoreovirus MyRV1 and MyRV3 were also previously reported to undergo genome alterations different from the previous examples, and we have observed the same phenomena with MyRV2 (B. Hillman and N. Suzuki, unpublished results). Fungal strains bearing MyRV3 isolates that have 12 genome segments lose S8 during subculturing of the original fungal isolate, generating a viral strain with a genome of 11 segments and no detectable remnant of S8 (Kanematsu et al., 2004). It is noteworthy that MyRV3 S8 has no counterpart in MyRV1 with a genome of 11 segments (Suzuki et al., 2004). In the case of MyRV1, intragenic rearrangements of MyRV1 S6 (in-frame duplication) and S10 (large internal deletion) are induced frequently by co-infection with the unrelated hypovirus CHV1 or transgenic expression of the multifunctional protein p29 encoded by CHV1 (Sun and Suzuki, 2008;
In this study, MyRV1 S4ss was found to be similar to S10ss in that large portions of its coding domain was deleted (Fig. 2), but different in that generation of S4ss was not associated with CHV1 p29.

Recently reverse genetics systems in which a whole set of genome segments or a particular genome segment is provided from cDNA clones have been developed for a few members of the family Reoviridae, including rotavirus, orthoreovirus and bluetongue virus (Boyce et al., 2008; Kobayashi et al., 2007; Komoto et al., 2006). Such plasmid-based approaches have contributed greatly to functional analyses of reovirus genomes (e.g., Celma and Roy, 2009; Kobayashi et al., 2009), but remain unavailable for most reoviruses. As an alternative, genetics with reassortments or rearrangements continue to prove useful and powerful for reoviruses. As an example, genetics with reassortments or rearrangements continue to prove useful and powerful for reoviruses (e.g., Ramig, 1997; Taniguchi and Urasawa, 1995). Our previous study (Sun and Suzuki, 2008) validated this approach and provided interesting insights into the functional roles of MyRV1 S6 and S10 in viral replication and symptom induction. MyRV1 S10 was shown to be dispensable for virus replication but a contributor to symptoms caused by MyRV1, whereas rearrangement of S6 had little overt effect on virus accumulation and symptom development. In contrast, rearrangements of MyRV1 genome segments encoding structural proteins such as VP1 result in reduced virus accumulation and altered symptom induction (Tanaka, Sun and Suzuki, unpublished results). The present findings indicate that infection by MyRV1/S4ss showed a phenotype similar to that of colonies infected by MyRV1/S4ss, rather than those infected by MyRV1/S10ss (Figs. 5 and 7).

Inability of viral genes to be expressed, as a result of spontaneous or artificially induced mutations, often results in impaired replication even if resulting virus may be viable. Therefore, it was surprising that MyRV1 strains unable to express VP4 (Fig. 4) or VP10 (Sun and Suzuki, 2008) were still viable, retaining replication levels comparable to that of wild-type MyRV1. It is more surprising that a virus strain lacking major portions of both VP4 and VP10, MyRV1/S4ss + S10ss, was able to replicate at similar levels to wild-type MyRV1 (Fig. 6B). In animal reoviruses, rearrangements of genomic segments encoding non-structural proteins occur much more frequently than those of segments encoding structural proteins. For example, rotavirus mutants able to express only a truncated form of non-structural protein NSP1 (Hua and Patton, 1994), or unable to express it at all (Taniguchi et al., 1996) are still viable despite its possible functional role as an antagonist of interferon-mediated, anti-virus responses (Barro and Patton, 2007). Rearrangements of plant reoviruses are also capable of replication in plant hosts (Nuss, 1984; Tomaru et al., 1997). To our knowledge, MyRV1/S4ss + S10ss is the first example of a reovirus that can replicate with deletions of over three fourths of the coding domains of two genome segments.

MyRV1/S4ss differs from wild-type MyRV1 in symptom induction properties and vertical transmission frequency, but levels of...
replication of the two viruses is similar. Mycoviruses infecting ascomycetous fungi are frequently transmitted through asexual spores at varying rates, depending on virus/host combinations (Enebak et al., 1994; Ghabrial and Suzuki, 2009). In C. parasitica, such transmission may or may not correlate with virus accumulation. Based on mutant analysis of different viral genes in the context of reverse genetics systems, asexual transmission frequency of the hypoviruses CHV1 appeared to correlate strongly with virus accumulation (Suzuki et al., 2003; Deng and Nuss, 2008). Elevation of MyRV1 replication levels by CHV1 p29 also resulted in an enhanced vertical transmission frequency of MyRV1 (Sun et al., 2006). The current study reveals that S4ss-containing MyRV1 variants are severely impaired, resulting in vertical transmission of less than 10% of the wild-type MyRV1 (Table 2). Given the comparable replication levels of S4ss-carrying strains to the wild-type strain (Fig. 3A), its extremely low transmission frequency is unlikely to be related to replication levels.

Asexual spores, or conidia of an ascomycetes such as C. parasitica develop from the side or tip of a highly specialized hypha called a conidiophore. These conidiophores hold chains of conidia, which are formed inside the fertile hyphae giving rise to a pycnidia or asexual fruiting bodies. Although it remains largely unknown how vertical transmission of mycovirus into conidia proceeds, it must achieve sufficient levels of accumulation in the conidiophore and conidium during conidiogenesis for its transmission. There might be a mechanism by which mycovirus cannot be present as a form able to initiate infection in the conidiophore or conidium, given that single-conidial isolation and hyphal tipping often cure mycovirus infection. Some viruses may not be able to move to the hyphal tip fast enough as the hypha grows. Alternatively, host fungi may actively eliminate virus during conidium formation. It is interesting to speculate that S4-coded VP4 may allow MyRV1 to efficiently spread to, and replicate in, conidia by an as-yet unknown mechanism.

MyRV1 VP4 is interesting from several perspectives. At the N-terminus, it possesses a myristoylation site and a presumptive autocleavage site (diptope N\(^{47}\)P\(^{48}\)) with notable similarities to the Mammalian orthoreovirus (MRV) \(\mu\)1 protein and VP4s of the coltiviruses, Eyach virus and Colorado tick fever virus. Sequence similarities in the remainder of MyRV1 VP4 showed that the coltivirus VP4s were homologous to MyRV1 VP4, but were insufficient to draw the conclusion that MRV \(\mu\)1 was their homologue (Suzuki et al., 2004). The MyRV1 VP4 aligns well overall with VP4s of the other fungal reoviruses, MyRV2 and MyRV3, but the MyRV3 VP4 shows no evidence for a putative myristoylation site (Suzuki et al., 2004). The \(\mu\)1 protein of MRV is particularly well characterized. It is a major outer capsid protein whose N-terminal myristoylation and proteolytic cleavage is required for membrane penetration during virus entry into host cells (Odegard et al., 2004; Tilloston and Shatkin, 1992) and is implicated in apoptosis of host cells upon infection (Danthi et al., 2008). Unlike MRV \(\mu\)1, MyRV1 S4-coded VP4 appears to be a non-structural protein that is detectable in infected fungal cells (Fig. 4), but not in purified virus preparations (T Tanaka and N Suzuki, unpublished results). Interestingly, all of the altered S4 segments in this study (S4ss1–S4ss4) have the potential to encode the N-terminal, putative myristoylation target C residue and to extend through the presumptive cleavage dipeptide, N\(^{47}\)P\(^{48}\), although these have not yet been detected in infected fungal spheroplasts (data not shown). It therefore remains unknown whether MyRV1/VP4 is myristoylated and cleaved in infected cells, and what the role of this protein is in the virus life cycle.

Materials and methods

Viral and fungal strains

The standard strain EP155 of C. parasitica infected with the type species, MyRV1 of the genus Mycoreovirus was described by Hillman et al. (2004a). MyRV1 with S10 genome rearrangements (S10ss2) had been described earlier by Sun and Suzuki (2008), while those with S4 rearrangements were obtained in this study (Table 1). EP155 transformed with the CHV1 p29 coding domain (Twtp29) was prepared previously (Sun et al., 2006; Suzuki et al., 2003). Culture and maintenance of the fungal strains were done in accordance with the method of Sun et al. (2006). Fungal colonies were grown for 5–10 days under bench top conditions at 24–26 °C on potato dextrose agar (PDA, Difco) or in potato dextrose broth (PDB, Difco).

Transformation of C. parasitica spheroplasts

Spheroplasts were prepared from C. parasitica EP155 infected with MyRV1 cultured in PDB using the methods of Eusebio-Cope et al. (2009). Transformation of resulting spheroplasts with the mutagenic plasmid pHygR was done as described previously (Faruk et al., 2008a, 2008b).
RNA extraction and Northern blot analysis

Total RNA at an optical density of 25 at 260 nm (OD260) was prepared from PDB cultures as described by Suzuki et al. (2003), entailing treatment with RQ1 DNase I (Promega, Madison, WI). For quantitative analysis, total RNA was separated in 1.4% agarose gel in 1× TAE [40 mM Tris/acetate (pH 7.8), 1 mM EDTA] buffer, stained with ethidium bromide, and viewed under a UV transilluminator. Single-stranded RNA was enriched using LiCl from total RNA fractions for Northern analysis. MyRV1 dsRNA was purified by the method of Sun et al. (2006) using CC41 cellulose column chromatography (Isogai et al., 1998).

For conventional Northern blotting 10 μg of ssRNA denatured in 1× MOPS buffer containing 3.7% formaldehyde and 42.5% formamide at 65 °C for 8 min, was separated by electrophoresis through 1.4% agarose gel containing 2% formamide and capillary-transferred onto Hybond-N+ nylon membranes (Amersham Biosciences, Buckingham, UK). Viral dsRNA genome segments were separated in SDS-polyacrylamide gels (12.5%) in Laemmli’s buffer system, and blotted onto Hybond-N+ nylon membrane in a submarine electroblot apparatus (Marsylo Model K58452) as described by Sun and Suzuki (2008). Irrespective of RNA type, the membrane was washed twice in 2× SSC (0.3 M NaCl, 0.03 M NaOAc, pH 7.0), twice in 0.1× SSC (15 mM NaCl, 1.5 mM NaOAc, pH 7.0), and subjected to UV cross-linking and probing with dioxigenin (DIG)-11-dUTP-labeled DNA fragments amplified by PCR in accordance with the method recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Probes specific for S1, S2, S4, and S10 were amplified by primer sets S1ORF1 and S1ORF2, S2FL1B and S2FL2B, and S4FL1 and S4FL2, and S10FL1 and S10FL2, which spanned positions 26–4090, 1–3846, 1–2269, and 1–975, respectively. Pre-hybridization, hybridization, and detection of hybridization signals were done as described earlier (Suzuki et al., 2003).

Western blot immunological analysis

Polyclonal antibodies against MyRV1 VP4 were prepared as described by Suzuki et al. (1994). The N (aa 1–349) and C terminal portions (aa 364–721) of VP4 were expressed as GST-fusion products in E. coli strain BL21 using an expression vector, pGEX-6P-1. Overexpressed polypeptides were purified using a GST column in accordance with the manufacturer’s protocol (Promega) and were used to immunize Japanese white rabbits by intramuscular injection (1st–5th) (1 mg/injection). A Sepharose affinity column (NHS-activated Sepharose 4 Fast Flow, Amersham Pharmacia Biotech) was employed to purify antibodies specific for VP4. Antisera against MyRV1 VP2 and VP10 have been described elsewhere (Tsutani et al., 2009; Sun and Suzuki, 2008).

Protein fractions for Western analysis were prepared basically as described by Sun and Suzuki (2008). Spheroplasts (approximately 5×10^7) were prepared by the method of Eusebio-Cope et al. (2009) and lysed in 1 ml of 50 mM Tris–HCl, pH 8.0, 75 mM NaCl by three rounds of freeze–thaw treatment using liquid nitrogen and 37 °C water. Proteins in the lysates were recovered as pellets after centrifugation at 1000 × g. The proteins were denatured, electrophoresed, and blotted as described by Suzuki et al. (1994), and detected using 0.033% NBT (nitro-blue tetrazolium chloride) and 0.0165% BCIP (5-bromo-4-chloro-3-indolyolphosphate p-toluidine) as substrates.

Determination of biological properties

Conidiation was measured by the method of Hillman et al. (1990). Four-day-old fungal isolates grown on a laboratory bench at 25–27 °C were later cultured for an additional 10 days under moderate light of approximately 3000 lx, and then assessed for their asexual sporulation. Each sporulating culture plate (60 cm) was flooded with 4 ml of water containing 0.15% Tween-20 and conidia were liberated using glass rods. The conidia were collected after sieving the suspension through two layers of Miracloth. Spore suspensions were prepared by serial dilution and conidial counts were assessed in each dilution with the aid of haemacytometer. All steps were done aseptically.

Efficiency at which each virus strain was transmitted through the conidia was assessed by plating out the spore suspension onto PDA. When the growth of germlings was evident, they were transferred to a new PDA plate and grown as described above. Infected fungal isolates were assessed initially by visual observation and their genotype was confirmed by Northern blot analysis.

For virulence assays, commercially available apples were inoculated with uninfected or virus–infected fungal cultures as described by Hillman et al. (2004). Lesion sizes (area) were assessed for each inoculated apple at 10 and 14 days post inoculation.

Sequence determination of rearranged segments

Smaller genome segments (S4ss1 to S4ss4) derived from S4 were separated in SDS–PAGE gels, and eluted from gel slices stained by EtBr as described by Suzuki et al. (1990). The eluted dsRNA was denatured in 90% DMSO for 15 min at 65 °C (Asamizu et al., 1985) and used as a template for cDNA synthesis and subsequent PCR (Sun and Suzuki, 2008). Resulting cDNA fragments were cloned into pGEM-T-easy (Promega). 5′-RACE using terminal deoxynucleotidyl transferase was carried out to confirm the terminal sequences of the segments, as described by Suzuki et al. (2004). These clones were sent to Macrogen Japan (Tokyo) for sequencing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.11.035.

References


