Rearrangements of mycoreovirus 1 S1, S2 and S3 induced by the multifunctional protein p29 encoded by the prototypic hypovirus Cryphonectria hypovirus 1 strain EP713

Toru Tanaka,1 Liying Sun,2† Kouhei Tsutani3 and Nobuhiro Suzuki1

1Agrivirology Laboratory, Institute of Plant Science and Bioresources, Okayama University, Kurashiki, Okayama 710-0046, Japan
2Zhejiang Provincial Key Laboratory of Plant Virology, Institute of Virology and Biotechnology, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, PR China

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

Received 2 February 2011
Accepted 12 April 2011

Mycoreovirus 1 (MyRV1), a member of the family Reoviridae possessing a genome consisting of 11 dsRNA segments (S1–S11), infects the chestnut blight fungus and reduces its virulence (hypovirulence). Studies have previously demonstrated reproducible induction of intragenic rearrangements of MyRV1 S6 (S6L: almost full-length duplication) and S10 (S10ss: internal deletion of three-quarters of the ORF), mediated by the multifunctional protein p29 encoded by the prototype hypovirus, Cryphonectria hypovirus 1 (CHV1) strain EP713, of the family Hypoviridae with ssRNA genomes. The current study showed that CHV1 p29 also induced rearrangements of the three largest MyRV1 segments, S1, S2 and S3, which encode structural proteins. These rearranged segments involved in-frame extensions of almost two-thirds of the ORFs (S1L, S2L and S3L, respectively), which is rare for a reovirus rearrangement. MyRV1 variants carrying S1L, S2L or S3L always contained S10ss (MyRV1/S1L+S10ss2, MyRV1/S2L+S10ss2 or MyRV1/S3L+S10ss2). Levels of mRNAs for the rearranged and co-existing unaltered genome segments in fungal colonies infected with each of the MyRV1 variants appeared to be comparable to those for the corresponding normal segments in wild-type MyRV1-infected colonies, suggesting that the rearranged segments were fully competent for packaging and transcription. Protein products of the rearranged segments were detectable in fungal colonies infected with S2L MyRV1/S2L+S10ss2 and S3L MyRV1/S3L+S10ss2, whilst S1L-encoded protein remained undetectable. S1L, S2L and S3L were associated with enhancement of the aerial hyphae growth rate. This study has provided additional examples of MyRV1 intragenic rearrangements induced by p29, and suggests that normal S1, S2 and S3 are required for the symptoms caused by MyRV1.

INTRODUCTION

Mycoreovirus 1 is the type species of the recently established genus Mycoreovirus within the subfamily Spinareovirinae (’turreted’ reoviruses) of the family Reoviridae, members of which have genomes composed of 11 dsRNA segments (S1–S11) ranging from 4127 to 732 bp in size (Hill et al., 1999; Hillman et al., 2004; Suzuki et al., 2004). Biochemical and bioinformatics analyses of mycoreovirus 1 (MyRV1) have suggested functional roles of some viral proteins such as VP1 (RNA-dependent RNA polymerase, RdRp), VP3 (guanylyltransferase) and VP6 (nucleoside triphosphate binding) (Hillman et al., 2004; Supyani et al., 2007; Suzuki et al., 2004). By analogy with other turreted reoviruses (Reinisch et al., 2000), VP1 (RdRp) and VP3 (capping enzyme) are assumed to be structural core proteins; however, experimental proof awaits further study. MyRV1 S10-encoded VP10 and S4-encoded VP4 seem to be non-structural proteins (Eusebio-Cope et al., 2010; Sun & Suzuki, 2008). The location of other viral proteins has yet to be elucidated. MyRV1 infects one of the most destructive tree-pathogenic fungi, the chestnut blight fungus, and reduces its virulence to the tree host (hypovirulence). However, the virus exerts few effects on host asexual sporulation and pigmentation, in contrast to other hypovirulence-causing viruses such as the prototype hypovirus, Cryphonectria hypovirus 1 strain EP713 (CHV1-EP713), of the family Hypoviridae, closely related to the
picornavirus-like superfamily, with ssRNA genomes (Koonin et al., 1991). Reverse genetics is an approach available only for limited reovirus species such as orthoreovirus, rotavirus and orbivirus (Boyce et al., 2008; Kobayashi et al., 2007; Komoto et al., 2006). This situation has greatly hampered analysis of the functional roles of the gene segments in the infection cycle of other reoviruses.

Genome rearrangements are commonly observed in all major genera of the family Reoviridae (Arella et al., 1988; Desselberger, 1996; Eaton & Gould, 1987; Maoka et al., 1993; Murao et al., 1996; Nuss, 1984; Schnepp et al., 2008; Taniguchi & Urasawa, 1995), contributing to functional analyses of their genome segments. Two types of rearrangement, inducible and spontaneous, are known to occur in MyRV1. A previous study of mixed infection of Cryphonectria parasitica by MyRV1-CPb21 and CHV1-EP713 (Sun et al., 2006) led to the discovery of an inducible rearrangement of MyRV1 mediated by the papain-like protease p29 of CHV1-EP713 (Sun & Suzuki, 2008; Sun et al., 2006). MyRV1 S6 and S10 were reportedly rearranged into S10ss (a 74.2–76.4 % deletion of the coding domain) and S6L (an in-frame duplication of the coding domain) at a very high frequency. Whilst MyRV1 S10-encoded VP10, dispensable for virus viability, is required for the induction of normal virus symptoms, no appreciable effects are associated with S6 rearrangements. Like the HC-Pro encoded by the family Potyviridae within the picornavirus-like superfamily (Kasschau & Carrington, 1998; Koonin et al., 1991; Maia et al., 1996), CHV1 p29 is multifunctional. Assigned roles include virus viability, symptom induction, elevation of replication and transmission of heterologous and homologous viruses, polyprotein processing and RNA silencing suppression (Choi et al., 1991; Craven et al., 1993; Segers et al., 2006; Sun et al., 2006; Suzuki et al., 1999, 2003). p29 is known to be co-fractionated with MyRV1 dsRNA and to bind MyRV1 VP9 in vivo when both p29 and MyRV1 are present in single fungal colonies (Sun & Suzuki, 2008). However, it remains largely unknown how p29 induces rearrangements of the distinct virus.

Spontaneous rearrangements of MyRV1 S4 were recently reported to occur in transformants carrying random integrations of a drug-resistance gene (Eusebio-Cope et al., 2010). These p29-independent rearrangements occur much less frequently than p29-dependent rearrangements. Rearranged segment S4ss contains deletions of large proportions (78–89 %) of the coding domains. Surprisingly, MyRV1 variants carrying S4ss (MyRV1/S4ss) and lacking normal S4 can still replicate well but show impaired vertical transmission via conidia. MyRV1/S4ss-infected colonies show a severe reduction in the aerial hyphae growth rate. More surprisingly, a reassortant carrying S4ss and S10ss is replication competent. These circumstances contribute to functional assignment of MyRV1-encoded proteins.

The present study represents an extension of our analysis of p29-mediated rearrangements aimed at clarifying the functions of MyRV1 proteins in virus replication and symptom expression. We have presented evidence that MyRV1 S1, S2 and S3 are rearranged in an intramolecular way by trans supply of p29 to extend their ORFs encoding structural proteins. It was confirmed that altered, longer versions of the viral proteins encoded by S2 and S3 were expressed in infected colonies. Comparative inspection of fungal colonies infected by MyRV1 variants with rearranged segments revealed that normal S1, S2 and S3 are required for normal symptom induction by the virus.

RESULTS

MyRV1 genome rearrangement in fungal colonies transformed by the CHV1-EP713 p29 coding domain.

We previously isolated MyRV1 variants with rearranged S6 and S10 that had been induced in fungal transformants (Twtp29) expressing CHV1 p29 (Sun & Suzuki, 2008). An expanded search of approximately 200 subcultures of Twtp29 infected by MyRV1 allowed detection of three distinct virus variants among those isolated previously (Fig. 1a, lanes 3–5). Each viral strain contained an unusual large and a small segment whose mobilities on SDS-PAGE were greater than that of S1 (designated S1L, S2L and S3L, respectively) and less than that of S11 (S10ss2). The smallest segment commonly found in the variants was identified as the previously reported S10ss2, as described below. These variants are referred to hereafter as MyRV1/S1L+S10ss2, MyRV1/S2L+S10ss2 and MyRV1/S3L+S10ss2, respectively.

Careful inspection of Fig. 1 showed that the band intensity of the authentic S1 in MyRV1/S1L+S10ss2 was weaker than that in wild-type MyRV1. Likewise, S2 of MyRV1/S2L+S10ss2 and S3 of MyRV1/S3L+S10ss2 showed a weaker intensity. This suggested that S1L, S2L and S3L originated from S1, S2 and S3, respectively. Moreover, the authentic S10 band was absent in all variant viral strains (Fig. 1, lanes 3–5).

S1L, S2L, S3L and S10ss originate from MyRV1 S1, S2, S3 and S10, respectively

To confirm the origins of the rearranged segments, Northern blotting was conducted. As shown in Fig. 1, S1L hybridized with an S1-specific probe, whilst S2L and S3L were detected by S2- and S3-specific probes, respectively. In addition to these unusual segments, normal S1, S2 and S3 were detected in RNA preparations from mycelia infected with MyRV1/S1L+S10ss2, MyRV1/S2L+S10ss2 and MyRV1/S3L+S10ss2, respectively (Fig. 1b–d). The smallest segment, S10ss2 hybridized to the S10 probe in the three MyRV1 variants, and no intact S10 was detected. From these results, the origins of S1L, S2L, S3L and S10ss were established as S1, S2, S3 and S10, respectively.
On the basis of the SDS-PAGE profile (Fig. 1a), an extension was expected to occur on each rearranged segment. To clarify the sequences of S1L, S2L and S3L, RT-PCR clones that spanned the extension junctions were obtained and sequenced. Primer pairs in which the forward and reverse primers corresponded to the 3′- and 5′-terminal regions of authentic segments, thus being unable to amplify fragments on normal segments, were chosen randomly. The positions and sequences of the primers used for RT-PCR are shown in Supplementary Table S1 (available in JGV Online). As a consequence, RT-PCR amplified DNA fragments of 1.6 kb from S1L, 1.3 kb from S2L and 0.5 kb from S3L (data not shown).

Sequencing of the RT-PCR clones revealed the genetic structures of the rearranged segments S1L, S2L and S3L. All were similar, carrying a head-to-tail, in-frame extension of the coding domain, as in the case of S6L (Sun & Suzuki, 2008). S1L consisted of two segments of normal S1 in which the first segment lacked a 3′-region of S1 corresponding to map positions 2719–4127, whilst the second segment contained a deletion of positions 1–387 (Fig. 2a). S1L had an extended ORF that would encode a protein of 2131 aa. In S2L, the ORF was enlarged by approximately 140% relative to the normal S2 ORF comprising 1238 codons (Fig. 2b). Extension end points were present at C2614 and C1133. S3L was a nearly duplicated form of the normal S3 ORF, although nt 2468–3258 of the first segment and nt 1–520 of the second segment were deleted (Fig. 2c). The modified ORF would encode a polypeptide of 1714 aa. These rearrangements of S1, S2 and S3 could be generated by single internal deletion events. Sun & Suzuki (2008) reported two versions of S10ss (S10ss1 and S10ss2) that differed slightly in size. The smaller segments contained in the three MyRV1 variants with rearrangements were shown to be S10ss2 by sequence analyses.

MyRV1 S1, S2 and S3 encode structural proteins

We previously detected a few possible structural proteins of approximately 130 and 65 kDa (Hillman et al., 2004). However, gene-product assignment for this virus has not been well established. Our previous studies showed that VP1 and VP3, encoded by S1 and S3, are the RdRp and guanylyltransferase, respectively (Supyani et al., 2007; Suzuki et al., 2004). All reoviruses for which genome-product assignment has been established incorporate these enzymes within core particles or the spikes extending from them. Using an immunological assay, we determined whether this was also the case for MyRV1.

We first purified MyRV1 particles using the method of Hillman et al. (2004) with a minor modification. As they reported, the purified preparation contained major components of approximately 130 and 65 kDa (Fig. 3a). Inspection of Fig. 3(a) confirmed the presence of minor 150 and 115 kDa components that were absent in the virus-free C. parasitica strain EP155 and were considered to
be structural proteins. To confirm this, we prepared antisera against viral proteins VP1, VP2 and VP3 and used them for Western blotting. The antibody against VP1 reacted with the 150 kDa polypeptide that co-migrated with recombinant VP1 expressed in insect cells via a baculovirus vector (Fig. 3b). The 130 and 115 kDa polypeptides also were detected by Western blotting with antibodies against recombinant VP2 and VP3, respectively (Fig. 3b).

Fig. 2. Diagrammatic representation of the organization of MyRV1 rearranged segments, S1L, S2L and S3L. The genetic organization of the normal and altered forms of genome segments S1 (a), S2 (b) and S3 (c) is shown. Coloured boxes indicate coding domains, whilst black bars indicate the untranslated regions (UTRs). The positions of the start and stop codons, and extension end points, are shown above and below the diagrams. Nucleotide and amino acid sequences adjacent to the altered sites on normal and rearranged segments are also shown. The authentic S1, S2 and S3 segments are 4127, 3846 and 3258 nt, possessing single large ORFs and relatively short 5′-UTRs (25–80 nt) and 3′-UTRs (33–49 nt). The rearranged segment S1 (S1L) is 6458 bp with an extension of almost the entire S1 segment. S1L retains the 5′-three-quarters of S1 (nt 1–2718) linked in frame to 84 % of the 3′-terminal portion (nt 388–4127). In S2L, two fragments spanning nt 1–2614 and 1133–3846 are fused, leading to an in-frame extension of the ORF, which would encode a protein of 1732 aa. Like S1L and S2L, S3L carries an in-frame ORF extension resulting from fusion of the 5′ segment (nt 1–2467) and the 3′ segment (nt 521–3258) of S3. No sequence heterogeneity was detected at the junction sites by sequence analysis of ten RT-PCR clones for each rearrangement.

Fig. 3. Western blot analysis of MyRV1 structural proteins. (a) SDS-PAGE analysis of MyRV1 structural proteins. Virus particles were purified by differential and sucrose density-gradient centrifugation from MyRV1-infected fungal colonies grown in PDB. Proteins in the lysates of insect cells infected with recombinant baculoviruses carrying genome segments of MyRV1 S1 (VP1), S2 (VP2) and S3 (VP3) (Supyani et al., 2007) were prepared as described by Matsuura et al. (1987). Proteins in purified virus preparations (MyRV1) and the insect lysates were resolved by SDS-PAGE (12.5 % acrylamide) and stained with silver. Protein fractions (lane EP155) obtained from virus-free EP155 by the same method as that used for virus purification were also analysed in this and subsequent Western blotting procedures. Note that the starting material for EP155 was fivefold greater in semi-dry weight than that for the virus-infected strain. Arrows indicate recombinant VP1, VP2 and VP3. M, protein size standards (Bio-Rad). (b) Western blot analysis of MyRV1 structural proteins. The same set of protein preparations as those used in (a) was probed with polyclonal antibodies against MyRV1 VP1, VP2 and VP3, as indicated. Molecular mass standards (10–250 kDa) available from Bio-Rad were used in this and subsequent experiments.

These results clearly showed that VP1, VP2 and VP3 are structural proteins.
MyRV1 S1L, S2L and S3L are expressed in fungal colonies infected with MyRV1

Northern blot analysis showed that the rearranged segments S1L, S2L and S3L were transcribed efficiently and that accumulation levels of their transcripts were comparable to those of normal transcripts in wild-type MyRV1-infected colonies (Fig. 4a). In addition to the rearranged transcripts, the equivalent normal-sized transcripts were detectable in fungal colonies infected with MyRV1 harbouring the rearrangements. However, the ratios of the lengthened to the normal transcripts varied among subcultures, as was the case for MyRV1/S6L (Sun & Suzuki, 2008). Transcripts derived from S10ss2, but not from S10, were observed in fungal colonies infected with each of the MyRV1 variants. These results suggested that each of the rearranged segments is packaged in core particles, which are believed to be the site of transcription.

In order to examine whether the proteins encoded by the rearranged segments were expressed, we attempted to detect the expected longer versions of VP1, VP2 and VP3 (VP1L, VP2L and VP3L) in total protein fractions from infected mycelia (expected sizes of 2131, 1732 and 1714 aa, respectively). VP1 was evident in all virus-infected fungal colonies, although its accumulation in those infected with MyRV1/S1L+S10ss2 appeared to be much less than in the other strains (Fig. 4b, top panel). We failed to detect VP1L in fungal colonies infected with MyRV1/S1L+S10ss2. A VP1-specific antibody purified using a Sepharose affinity column did not allow detection of VP1L. In addition to VP2 (130 kDa) and VP3 (115 kDa), VP2L and VP3L were detected at the expected positions (~190 kDa) and were absent in MyRV1-infected fungal stains (Fig. 4b, second and third panels, respectively).

Phenotype of fungal colonies infected with MyRV1 variants with rearranged segments

MyRV1/S1L+S10ss2, MyRV1/S2L+S10ss2 and MyRV1/S3L+S10ss2 were generated in the Twp29 background expressing CHV1 p29. A transgenic supply of CHV1 p29 affects MyRV1 symptom induction and replication (Sun & Suzuki, 2008). Thus, it was necessary to examine the possible phenotypic effects of rearranged segments alone in the absence of p29. Taking advantage of the property that the extended large segments were relatively stably maintained without p29 transgenic expression, MyRV1 variants were transferred to EP155 non-transformants via anastomosis. Another complicating factor was the presence of S10ss2 in all the variants, which influenced the phenotype of MyRV1-infected fungal colonies (Sun & Suzuki, 2008). Therefore, we included the MyRV1/S10ss2-infected fungal strain as a reference.
Infection of EP155 by wild-type MyRV1 resulted in severely reduced growth of aerial hyphae and enhanced the production of brown pigment, whilst virus-free EP155 manifested vigorous mycelial growth and produced orange pigment (Fig. 5a). As reported earlier (Sun & Suzuki, 2008), EP155 infected with MyRV1/S10ss2 showed enhanced growth of aerial hyphae relative to MyRV1-infected colonies (Fig. 5a). Fungal strains infected with the three variants MyRV1/S1L+S10ss2, MyRV1/S2L+S10ss2 and MyRV1/S3L+S10ss2 commonly showed a phenotype similar to that of the MyRV1/S10ss2-infected strain on potato dextrose agar (PDA) plates, i.e. slightly enhanced growth of aerial hyphae (Fig. 5a). We also used Vogel’s N medium because it occasionally produces differences between C. parasitica strains that manifest similar phenotypes on PDA (N. Suzuki, unpublished data). On Vogel’s N medium, differences were also found between fungal colonies infected with viral strains (Fig. 5b). In comparison with virus-free EP155, wild-type MyRV1-infected colonies showed reduced growth but increased orange pigmentation. Infection of EP155 by MyRV1/S10ss2 resulted in a slight restoration of growth but a reduction in pigmentation. A further increase in growth and a decrease in pigmentation were observed in fungal colonies infected with the MyRV1 variants bearing S1L, S2L or S3L.

The levels of virulence of the fungal strains to chestnut were assessed using apples in which they could be measured by the size of the lesions induced by fungal colonies on apple fruits. Virus-free EP155 induced large lesions of ~37 cm² (mean value) in apples, whereas MyRV1-infected colonies induced lesions of ~1 cm² (Fig. 5c). As reported previously (Sun et al., 2006), EP155 infected with MyRV1/S10ss2 induced measurably larger lesions of approximately 5 cm² relative to the extremely small lesions induced by wild-type MyRV1-infected colonies. Lesions induced by fungal colonies infected with the MyRV1 variants were similar to or slightly larger than those induced by MyRV1/S10ss2-infected strains.

These combined results suggested that the rearrangements of S1, S2 and S3 are associated with phenotypic alterations of fungal colonies infected with MyRV1, including an increase in the growth of aerial hyphae.

The ratio of S1 : S1L transcripts is associated with symptoms induced by MyRV1 variants

Phenotypic alterations were further confirmed to be associated with the rearranged segments using MyRV1/S1L+S10ss2 with varying ratios of normal to altered S1 transcripts. Three substrains of MyRV1/S1L+S10ss2 (MyRV1/S1L+S10ss2-1, -2 and -8) were obtained that contained much greater, nearly equal and much reduced amounts of S1 transcripts relative to S1L, respectively (Fig. 6a). These substrains caused different degrees of reduction in aerial hyphae growth (Fig. 6b, reflected by the white mycelia). With increasing ratios of S1 : S1L transcripts, the substrains repressed more of the growth of the aerial mycelia (Fig. 6b). Fungal colonies infected with MyRV1/S1L+S10ss2-1 were morphologically indistinguishable from MyRV1/S10ss2-infected colonies, whilst fungal colonies...
mediated phenotype.

generated in C. parasitica promiscuously in generation of recombinants or selection on S6 and S10, and the data suggest that p29 is involved besides the previously characterized MyRV1 rearrangements. This study has thus provided additional examples p29 (Twtp29) and characterized biologically and molecularly. In this study, MyRV1 variants with rearrangements of the three largest segments, S1 (MyRV1/S1L + S10ss2), S2 (MyRV1/S2L + S10ss2) and S3 (MyRV1/S3L + S10ss2), were generated in C. parasitica transformants expressing CHV1 p29 (Twp29) and characterized biologically and molecularly. This study has thus provided additional examples of CHV1 p29-dependent MyRV1 genome rearrangements, besides the previously characterized MyRV1 rearrangements on S6 and S10, and the data suggest that p29 is involved promiscuously in generation of recombinants or selection of pre-existing rearranged segments that are tolerable for and/or advantageous to virus viability. The rearrangements on the three segments were similar to one another in molecular configuration, and also to S6L, which was characterized previously (Sun & Suzuki, 2008). All of these rearrangements entailed in-frame intragenic ORF extensions of >1.5-fold relative to the corresponding normal segments, and could be generated by single internal deletion events (Fig. 2).

Using immunological methods, MyRV1 S1, S2 and S3 were shown to encode three structural proteins, VP1, VP2 and VP3. VP2 is one of the major structural proteins, whilst VP1 and VP3 are considered to be minor structural proteins (Fig. 3). VP1 harbours the motifs of the RdRp (Hillman et al., 2004), whilst VP3 has a dihistidine motif characteristic of guanylyltransferases from the turreted group of the family Reoviridae, such as the genera Oryzavirus, Fijivirus, Orthoreovirus, Mycoreovirus, Avianreovirus and Cypovirus (Supyani et al., 2007). Although on the basis of their expected functions VP1 and VP3 are considered to reside within the core and to constitute the core spike (Reinisch et al., 2000), determination of their location in other turreted reoviruses awaits further experiments.

Rearrangements are frequently found in genome segments encoding non-structural proteins in the family Reoviridae. Modifications of these segments seem better tolerated than rearrangements on segments encoding structural proteins, probably because reovirus structural proteins play pivotal roles in fundamental key steps of the virus replication cycle such as RNA synthesis, virus entry and virion formation. In this respect, the rearrangements of MyRV1 S1, S2 and S3 are different from many other reovirus rearrangements reported previously. A limited number of examples of structural protein gene rearranged have been reported in the genera Phytoreovirus and Rotavirus. In plant reoviruses, some rearranged segments can be generated by maintaining them exclusively in one of the alternate hosts, either the plant or the insect vector. For example, wound tumour virus S1, S2, S5 and S7 are also known to be altered and encode structural (S1, S2 and S5) and non-structural (S7) proteins, often resulting in defects in vector transmissibility (Nuss, 1984). Another phytoreovirus, rice dwarf virus (RDV), also accumulated rapidly dysfunctional mutations in S2 and S10 when virus populations were maintained in plant hosts (Pu et al., 2011). RDV S2-encoded P2 is a major outer capsid protein involved in attachment to leafhopper cells. It should be noted that these modifications are deletions or nonsense mutations, rather than ORF extensions. A variety of rotavirus rearrangements, mostly intragenic, have been reported in both naturally infected individuals and laboratory-cultured host cells (Cao et al., 2008; Desselberger, 1996; González et al., 1989; Kojima et al., 2000; Patton et al., 2001; Schnepf et al., 2008; Taniguchi & Urasawa, 1995; Taniguchi et al., 1996). Interestingly, most rotavirus extensions are found down-stream of the authentic stop codons of genome segments encoding non-structural proteins, leading to expression of unaltered proteins. Exceptions include rotavirus S7, which

infected with MyRV1/S1L + S10ss2-8 restored, although not to the level of virus-free EP155, the growth of aerial hyphae (compare the centres of colonies infected by MyRV1/ S1L+ S10ss2-1 and MyRV1/S1L+ S10ss2-8). Colonies infected with MyRV1/S1L+ S10ss2-2 manifested an intermediate phenotype.

**Fig. 6.** Association of symptom expression and ratio of S1:S1L transcripts in colonies infected with MyRV1/S1L+ S10ss2. (a) Colony morphology of fungal strains infected with different substrains of MyRV1/S1L+ S10ss2. Fungal colonies infected with three substrains, MyRV1/S1L+ S10ss2-1, MyRV1/S1L+ S10ss2-2 and MyRV1/S1L+ S10ss2-8, were cultured on PDA for 1 week and then photographed. (b) Northern blotting of total RNA isolated from the fungal colonies infected with the MyRV1/ S1L+ S10ss2 substrains. Total RNA fractions were isolated from the fungal colonies used in (a), electrophoresed under denaturing conditions and subjected to Northern blotting as described in Methods.

**DISCUSSION**

In this study, MyRV1 variants with rearrangements of the three largest segments, S1 (MyRV1/S1L + S10ss2), S2 (MyRV1/S2L + S10ss2) and S3 (MyRV1/S3L + S10ss2), were generated in C. parasitica transformants expressing CHV1 p29 (Twtp29) and characterized biologically and molecularly. This study has thus provided additional examples of CHV1 p29-dependent MyRV1 genome rearrangements, besides the previously characterized MyRV1 rearrangements on S6 and S10, and the data suggest that p29 is involved promiscuously in generation of recombinants or selection of
is able to express a larger version of NSP3 (Gault et al., 2001).

In contrast to many previously reported rearranged segments with extensions, the MyRV1 rearranged segments (S1L, S2L and S3L) had enlarged ORFs capable of being transcribed and expressed (at least for VP2L and VP3L; Fig. 4). As noted for MyRV1/S6L (Sun & Suzuki, 2008), Northern blot analyses (Fig. 4a) showed accumulation of viral transcripts in fungal colonies infected with the MyRV1 variants comparable to those of wild-type MyRV1 or MyRV1/S10ss. Reoviral transcription is assumed to occur in the core. Therefore, the altered MyRV1 segments were fully functional in packaging and in RNA synthesis as templates. This finding provides another interesting insight into the size limitation of the core particle cavity. Reovirus core particles are considered to have a common structural architecture of T=1 (Grimes et al., 1998; Nakagawa et al., 2003; Reinisch et al., 2000). On the basis of examination of rotavirus rearrangements with extensions, it was postulated that an additional ~1.9 kb is maximal for this capacity (Gault et al., 2001; Hundley et al., 1987; Troupin et al., 2010). S1L, with the largest extension, was over 2.3 kb longer than the standard S1, and S10ss2 was 569 bp shorter than S10. Thus, the entire genome size of MyRV1/S1L+S10ss2 exceeded that of the wild-type by approximately 1.8 kb, within the range observed for rotaviruses. Determination of whether the altered segments and encoded proteins (VP1L, VP2L or VP3L) are packaged into virus particles has been hampered by difficulty purifying them, although this is definitely an interesting future challenge.

It may be premature to discuss the functions of the extended forms of structural proteins (VP1L, VP2L and VP3L) in RNA replication. However, a few observations suggest that these extended viral proteins are indeed functional. The MyRV1/S1L+S10ss2 and MyRV1/S3L+S10ss2 subarrays carrying excessive amounts of rearranged segments relative to the normal ones were still replication competent (Fig. 4a). If the rearranged segments were dysfunctional, virus replication would be impaired. Importantly, the functional motifs for RdRp (VP1) and guanylyltransferase (dihistidine; VP3) were duplicated in VP1L and VP3L. Furthermore, although VP1L could not be detected, expression of VP2L and VP3L was confirmed (Fig. 4b). The many unsuccessful attempts to detect VP1L may have been due to its inefficient transfer to the membrane and/or an insufficient titre of the VP1-specific antibody used in Western blotting. Functional interpretation of such results is undoubtedly complicated by the mixed accumulation of altered and unaltered segments (Fig. 1), allowing the possibility of trans-complementation by co-existing normal forms of the VP1, VP2 and VP3 proteins.

Interestingly, S10ss2 was always found together with S1L, S2L and S3L (Fig. 1). S10-encoded VP10, whilst dispensable for virus replication, contributes to the reduction in both aerial hyphae growth and virulence caused by MyRV1 (Sun & Suzuki, 2008). Therefore, determination of phenotypic alterations associated with S1L, S2L and S3L required comparisons among fungal strains infected with wild-type MyRV1, MyRV1/S10ss and each of the MyRV1 variants reported in this study (Fig. 5a, b). The rearrangements of S1, S2 and S3 were associated with similar phenotypic alterations, i.e. increased mycelial growth on PDA and Vogel’s N medium (Fig. 5a, b). Lesions induced by colonies infected with MyRV1 variants were significantly larger than those induced by colonies infected with wild-type MyRV1 but were comparable to those induced by MyRV1/S10ss. In the case of MyRV1/S1L, the symptom severity may be associated with the accumulated ratio of rearranged to unaltered segments. As the ratio of S1L:S1 increased, greater growth of aerial hyphae was observed in fungal colonies infected with MyRV1/S1L+S10ss2 (Fig. 6).

Unlike rotaviruses (Komoto et al., 2006; Trask et al., 2010), orthoreoviruses (Kobayashi et al., 2007) and orbiviruses (Boyce et al., 2008; Matsuo et al., 2010), reverse genetics using a complete or partial set of cDNAs of the genome segments is unavailable for many reoviruses, including MyRV1. However, our studies have opened up an alternative new avenue for investigation of MyRV1 proteins using spontaneous (Eusebio-Cope et al., 2010) and induced (Sun & Suzuki, 2008) rearrangements, and have clarified the functional roles of MyRV1 proteins. For example, it has been revealed that MyRV1 VP4 is required for efficient vertical transmission of the virus and normal expression of symptoms but is dispensable for efficient virus replication. Furthermore, 75% of the VP10-coding domain appears to be non-essential for virus replication. This study has provided additional examples of MyRV1 rearrangements induced by CHV1 p29, and these were utilized for functional analysis. We found that a total of six genome segments of MyRV1 underwent either induced or spontaneous rearrangement, contributing to functional assignment of the MyRV1 segments (Eusebio-Cope et al., 2010; Sun & Suzuki, 2008).

The CHV1 p29-mediated induction of MyRV1 genome rearrangements is an interesting phenomenon, but its molecular mechanism remains unknown. It is possible that p29 is directly involved in the production of rearrangements via an RdRp-associated template switch (Lai, 1992; Nagy & Simon, 1997) or RNA ligase-mediated events (Chetverin, 1999), or via enhanced selection of rearranged segments in infected colonies by changing the physiological cellular state. Sun & Suzuki (2008) revealed that CHV1 p29 shows specific interactions with MyRV1 VP9 and is cofractionated with MyRV1 dsRNA when both are present in fungal colonies. Furthermore, CHV1 p29 is multifunctional, serving as an RNA silencing suppressor, and playing roles in viral protein processing, symptom determination and replication enhancement of homologous and heterologous viruses. Further research to determine which activities of p29 are involved in the induction of MyRV1 rearrangements is now under way.
METHODS

Fungal strains, culture and virulence assay. C. parasitica strain EP155 was used as the host background. EP155 transformed with the coding domain of CHV1-EP713 p29 has been described previously (Sun & Suzuki, 2008; Sun et al., 2006). The MyRV1-harbouring fungal strain 9B21, belonging to a vegetative compatibility group different from EP155, was obtained from W. MacDonald (West Virginia University, WV, USA) and B. I. Hillman (Rutgers University, NJ, USA). An EP135 strain inoculated with purified virus particles of MyRV1-Cp9821 has been described previously (Hillman et al., 2004). For extraction of nucleic acid and protein, fungal colonies were grown in potato dextrose broth (PDB) (Difco) at a temperature of 24–26 °C. For phenotypic comparison, fungal strains were cultured on PDA (Difco) or Vogel’s N medium (Vogel, 1956).

Virulence levels of fungal colonies were estimated by assay with apple fruits, as described previously (Hillman et al., 2004). Apples were inoculated with plugs of freshly grown mycelia and incubated for 7–10 days at 25 °C.

Sequence determination of rearranged segments. Rearranged segments were eluted from SDS polyacrylamide gels as described by Suzuki et al. (1990). The isolated dsRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase after being denatured in 90 % DMSO for 15 min at 65 °C (Asamizu et al., 1985). dsDNAs complementary to different portions of the rearranged segments were amplified by RT-PCR with different combinations of primer sets and cloned into pGEM-T Easy (Promega). Purified plasmid clones were sent to Macrogen for sequencing.

RNA preparation and Northern blot analysis. Total RNA was prepared from C. parasitica mycelia cultured in 50 ml PDB by the method of Suzuki & Nuss (2002), which entailed treatment with RQ1 RNase-free DNase I (Promega), followed by extraction with phenol, phenol/chloroform and chloroform, and precipitation with ethanol. ssRNA fractions were enriched by 2 M LiCl precipitation. dsRNA was isolated from total RNA fractions using CCA1 cellulose (Whatman) column chromatography.

Conventional Northern blotting was performed as described by Suzuki et al. (2003). ssRNA was electrophoresed in a 1.4 % agarose gel under denaturing conditions, and then capillary transferred onto a nylon membrane (Hybond N+ nylon membrane (GE Healthcare)). Viral genomic dsRNA was resolved by SDS-PAGE (12 % acrylamide) in a Laemmli buffer system (Laemmli, 1970). After washing in 1 x TBE (pH 8.3), the gel was subjected to electrotransfer in a submarine electroblot apparatus (Marysol model KS8452) at a constant current of 400 mA for 5 h and denatured as described above.

Washing, pre-hybridization and hybridization were carried out as described by Sun & Suzuki (2008). The RNA was then probed with digoxigenin-11-dUTP-labelled DNA fragments prepared as recommended by the manufacturer (Roche Diagnostics). Chemiluminescent signals were visualized on a film.

Western blot analyses. Polyclonal antibodies to MyRV1 VP1, VP2 and VP3 were prepared basically as described previously (Sun & Suzuki, 2008; Suzuki et al., 1994). Truncated forms of MyRV1 VP1 N-terminal (aa 1–333) and C-terminal (aa 742 –1354) regions were expressed as fusion proteins in Escherichia coli using the expression vector pGEX-6P-1 and purified according to the manufacturer’s protocol. VP2 and VP3 were expressed in insect cells via baculovirus vectors (Supyani et al., 2007) and purified from SDS-polyacrylamide gels as described by Suzuki et al. (1994). The recombinant proteins (1 mg per injection) were used to immunize Japanese white rabbits by intradermal (first and second) and intramuscular (third to fifth) injections.

Protein preparation for Western blotting has been described elsewhere (Sun & Suzuki, 2008). Western blotting was performed according to the method of Suzuki et al. (1994).

Virus purification. Mycelia infected with MyRV1 were homogenized in liquid nitrogen and mixed with 4 vols extraction buffer (0.1 M sodium phosphate, pH 7.0). After clarification with 20 % CCl4 or Vertrel XF (DuPont-Mitsui Fluorochemicals), the homogenates were centrifuged at 2200 g for 20 min. After addition of NaCl and PEG 6000 to final concentrations of 0.3 M and 6 %, respectively, the supernantant was stirred for 1 h at 4 °C, followed by centrifugation at 3600 g for 20 min. The resultant pellets, resuspended in 0.05 M sodium phosphate buffer (pH 7.0), were subjected to differential centrifugation and sucrose density gradient (20–50 %) centrifugation (Hillman et al., 2004). Virus particles were recovered from the middle fractions by recentrifugation at 66 000 g for 2 h.

ACKNOWLEDGEMENTS

The authors are grateful to Yomogi Inc. (to N.S.), the Sasakawa Scientific Research Grant from the Japan Science Society (to L.S.) and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-Oriented Industries (to N.S.) for financial support during this study. We wish to acknowledge generous gifts of fungal strains and plasmid clones from Drs Bradley I. Hillman (Rutgers University, NJ, USA), William MacDonald (West Virginia University, WV, USA) and Donald L. Nuss (University of Maryland, MD, USA).

REFERENCES


but necessary for efficient vertical transmission and normal symptom induction. *Virology* 397, 399–408.


Suzuki, N., Supyani, S., Maruyama, K. & Hillman, B. I. (2004). Complete genome sequence of Mycoreovirus-1/Cp9B21, a member of...


