Effects of Defective Interfering RNA on Symptom Induction by, and Replication of, a Novel Partitivirus from a Phytopathogenic Fungus, *Rosellinia necatrix*

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A novel mycovirus termed *Rosellinia necatrix* partitivirus 2 (RnPV2), isolated from a phytopathogenic fungus, *Rosellinia necatrix* strain W57, was molecularly and biologically characterized in both natural and experimental host fungi. Three double-stranded RNA (dsRNA) segments, dsRNA1, dsRNA2, and defective interfering dsRNA1 (DI-dsRNA1), whose sizes were approximately 2.0, 1.8, and 1.7 kbp, respectively, were detected in W57. While the dsRNA2 sequence, encoding the coat protein, was reported previously, dsRNA1 and DI-dsRNA1 were shown to encode competent and defective (truncated) RNA-dependent RNA polymerase, respectively. Artificial introduction of RnPV2 into an RNA silencing-defective, Dicer-like 2 knockout mutant (Δdcl-2) of a nonnatural host, *Cryphonectria parasitica* (chestnut blight fungus), resulted in successful infection by the DI-dsRNA1-carrying and -free RnPV2. The DI-dsRNA1-free RnPV2 strain was characterized by a higher ratio of accumulation of the intact dsRNA1 to dsRNA2, enhanced replication and severer symptom expression, compared with the DI-carrying strain. These findings confirmed the nature of DI-dsRNA1 as a DI-RNA. Both viral strains replicated to higher levels in a Δdcl-2 mutant than in a wild-type *C. parasitica* fungal strain (EP155) and induced severe symptoms in the Δdcl-2 mutant but subtle symptoms in EP155, indicating that the host RNA silencing targets the partitivirus. No obvious phenotypic effects of infection by either virus strain were detected in the natural host fungus. These combined results represent the first example of a partitivirus with DI-RNA that alters viral symptom induction in a host-dependent manner.

Defective RNA (D-RNA) and defective interfering RNA (DI-RNA) are forms of subviral RNAs detected widely among a variety of RNA viruses that occur naturally and/or during maintenance in the laboratory (1–3). D-RNA or DI-RNA has closely related, shorter forms of parental viral RNAs that usually encode truncated, defective proteins or no proteins. DI-RNAs impair replication of intact cognate viral RNAs, while D-RNAs do not. DI-RNAs are nonessential for virus replication but competent as replication templates relying on their parental helper viruses. It is widely accepted that DI-RNAs are generated de novo by an RNA-dependent RNA polymerase (RdRp)-mediated template switch mechanism. DI-RNAs generally have negative effects on symptom expression and replication of their helper viruses. There are a few hypotheses to account for DI-RNA-mediated reduction of helper virus replication levels and symptom induction (1–3). A widely accepted explanation is that competition with helper viruses over trans-acting factors and substrates for their replication may cause interference. As another model, DI-RNAs efficiently trigger, but are not targeted by, RNA silencing that degrades helper viral mRNAs as an antiviral defense mechanism in plants, leading to symptom attenuation (4, 5). Another interesting link between RNA silencing and DI-RNAs has been noted by Nuss and coworkers (6): RNA silencing is associated with the generation of DI-RNA in fungal cells infected with a hypovirus with a (+) single-stranded RNA (ssRNA) genome. They hypothesized that hypovirus RNAs fragmented by the host RNA silencing pathway increase the rate of, and provide substrate for, template switching to produce DI-RNAs.

The family *Partitiviridae* comprises four genera, *Partitivirus*, *Alphacryptovirus*, *Betacryptovirus*, and recently established *Crypsovirus*, that infect fungi, plants, plants, and protozoa, respectively (7). Members of this family possess bisegmented double-stranded RNA (dsRNA) genomes, each encoding RdRp and the coat protein (CP). This virus family, regardless of its host, generally shows latent infections. Exceptions include radish yellow edge virus occasionally inducing mild symptoms in Japanese radish (8), *Aspergillus* fumigatus partitivirus-1 (AfuPV-1) altering the colony morphology of the host fungus (9), and *Flammulina* velutipes browning virus (FvBV) that modulates the cap color of fruiting bodies in its host mushroom (10). It has been reported that some members additionally harbor satellite-like dsRNA segments either carrying or lacking a potential open reading frame (ORF) (11). However, unlike other dsRNA viruses, such as mycovirus (genus *Mycovirus*), for which DI-RNAs have been reported (12, 13), partitiviruses have never been reported to carry DI-RNA species accompanied by a helper virus. Because of the unavailability of reverse genetics for, and the artificial introduction of, most partitiviruses, no clear picture of interactions among subviral RNAs, their helper viruses, and hosts has emerged. Therefore, very few cause-effect relationships have been established for partitivirus infection.

*Rosellinia necatrix* is the causal agent of white root rot disease, which is one of the most devastating diseases occurring in peren...
nial plants worldwide (14, 15). As in some other phytopathogenic fungi such as the chestnut blight fungus, *Cryphonectria parasitica* (16) and the white mold fungus, *Sclerotinia sclerotiorum* (17), *R. necatrix* hosts a variety of viruses, including partitiviruses (18–20). For example, a field hypovirulent fungal strain, W8, was shown to be infected with a partitivirus *Rosellinia necatrix partitivirus 1* (RnPV1) (21) and a novel megabirnavirus, *Rosellinia necatrix megabirnavirus 2* (RnMBV2) (A. Sasaki, personal communication). Transfection with purified RnPV1 particles showed that RnPV1 alone conferred neither hypovirulence nor any symptoms (22). During the course of molecular characterization of the *R. necatrix*-infecting viruses, we found another partitivirus termed Rosellinia necatrix partitivirus 2 (RnPV2) with three genomic elements of dsRNA, dsRNA1, dsRNA2, and dsRNA1-derived DI-dsRNA1, carrying an internal deletion. The complete sequence of one of the dsRNA segments encoding CP (dsRNA2) was reported previously (23). A BLAST search with CPs of RnPV2 and a related partitivirus led to the discovery of multiple independent integrations of partitivirus CP genes into plant genomes (23, 24).

In the present study, applying a transfection technique with purified virus particles that had recently been developed for a partitivirus (22), we were able to obtain a DI-free strain of RnPV2 in an RNA silencing-defective mutant of a heterologous fungus, *C. parasitica* (6). We report here the molecular and biological properties of RnPV2, focusing on the effects of DI-dsRNA on replication and symptom induction using both natural and experimental host fungi. This report presents the first example of a partitivirus harboring a DI-RNA altering viral symptom induction and replication in a host-dependent manner and that is targeted by a host antiviral defense system, RNA silencing.

**MATERIALS AND METHODS**

**Fungal strains and viruses.** A virus-infected fungal strain, *R. necatrix* W57 (25), was isolated in Iwate Prefecture, Japan (Table 1). Complete sequences of the viral genome were determined and deposited in the EMBL/GenBank/DDJB databases under the accession numbers AB569997 for dsRNA1, AB569998 for dsRNA2 (23), and AB569999 for DI-dsRNA1. RnPV1 from the *R. necatrix* W8 strain (21) was used as a counterpart partitivirus. The *R. necatrix* W57-T25 strain, which is isogenic to W57 but virus cured by hyphal tipping (Fig. 1A), was used as the reference strain and as the transfection recipient (Table 1). *C. parasitica* standard strain EP155 and a *dcl*-2 mutant strain (Dicer-like 2 knockout mutant) (26) were used for RnPV2 transfection as well (Table 1). All fungal materials were maintained on potato dextrose agar (PDA; Difco Becton) plates and cultured in potato dextrose broth (PDB; Difco Becton) liquid medium for RNA sample preparation. For phenotype observations, PDA plates inoculated with *R. necatrix* were cultured for a week at 22°C in darkness while *C. parasitica* plates were cultured for 1 to 3 weeks at 24 to 28°C on a benchtop.

**Virus particle purification and electron microscopy.** The virus particle fraction was obtained as described by Lin et al. (20). Mycelia of fungal strain W57 were harvested from 2 liters of liquid PDB culture grown on a benchtop for 2 weeks, homogenized in the presence of liquid nitrogen, and then mixed with extraction buffer (0.1 M sodium phosphate, pH 7.0, containing 0.1% [wt/vol] β-mercaptoethanol), followed by clarification with carbon tetrachloride. By differential centrifugation, the semipurified virus par-
TABLE 1 Fungal and viral strains used in this study

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<tr>
<th>Strain</th>
<th>Description</th>
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<tr>
<td><strong>Fungal</strong></td>
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<tr>
<td>W57</td>
<td>Rosellinia necatrix field strain carrying RnPv2-W57</td>
<td>This study, 25</td>
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<td>W57-T25</td>
<td>Virus-cured strain derived from W57 via hyphal tipping (virus free)</td>
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<td>Standard strain of Cryptonectria parasitica (virus free)</td>
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<td>Δdcl-2 mutant</td>
<td>dcl-2 knockout mutant of EP155 (RNA silencing defective, virus free)</td>
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We analyzed the 54-kDa protein detected by SDS-PAGE (Fig. 1C) by PMF using MALDI-TOF MS and subsequent MS/MS. The result unequivocally showed that the major structural protein of RnPv2 was encoded by dsRNA2 (see Table S2 in the supplemental material).

DI-dsRNA1 (1,719 nt) appeared to be a DI-RNA of dsRNA1, and the RdRp-coding ORF was internally terminated by a frame shift due to a single nucleotide insertion; also, there was a 266-nt deletion in the central region of the segment (Fig. 1E). A number of nucleotide substitutions in DI-dsRNA1 were also found. Together with the fact that the sequenced dsRNA was from W57 subcultured only three times from the original stock, this suggested the occurrence of natural DI-dsRNA1 in the field before the isolation of this strain and not in the laboratory. Furthermore, DI-dsRNA1 may have been attributed to much less accumulation of dsRNA1 (Fig. 3A). Using Northern analysis of RnPv2 mRNAs, the ratio of the dsRNA1 transcript to that of DI-dsRNA1 was shown to be 1:12 to 15 (Fig. 3B), apparently supporting the above notion.

Phylogenetic placement of RnPv2 in a group comprising both plant and fungal members. We constructed an ML phylogenetic tree using a multiple alignment of RdRps from representative partitiviruses together with alphacryptoviruses and a crypsovirus. The resultant ML tree demonstrated that RnPv2 RdRp clusters with those sequences from other partitiviruses, such as oyster mushroom isometric virus II and Vicia faba partitivirus 1 (Fig. 4). Interestingly, this clade involves members of fungal partitiviruses (Partitivirus III, categorized in this study) and plant-infecting alphacryptovirus (Alphacryptovirus I) members, as previously reported (11). Typical fungal partitiviruses formed two groups (Partitivirus I and II) separately from the former group and a group of plant-infecting members which are presumed to possess double CP coding segments arises (Alphacryptovirus II). Note that these subgroups were specified in 9th report of the International Committee on Taxonomy of Viruses (7), except for the Partitivirus III subgroup.

Transfection of the heterologous fungus C. parasitica results in the occurrence of a DI-dsRNA1-free strain of RnPv2. We attempted to transfect C. parasitica (as an experimental host) with...
purified RnPV2 particles (Fig. 1B) by using a conventional method established previously (18, 29). A Dicer homologue del-2 knockout strain of *C. parasitica* (Δdel-2 mutant) (26) defective in RNA silencing-based antiviral defense was used. The rationale behind this experiment was that RNA silencing was shown earlier to play a pivotal role in the generation of DI-RNA of an ssRNA virus, Cryphonectria hypovirus 1 (CHV1) (Table 1)(6). Consequently, RnPV2 was able to infect the Δdel-2 mutant and—interestingly—two types of transfectants (C15 and C16 strains) different in their electrophoretic patterns of genomic RNA were obtained (Table 1). However, its transfection efficiency was low and only 2 out of 24 subcultures, derived from two regeneration plates 9 cm in diameter, were infected. The C15 transfectant strain showed an electropherotype very similar to that of the W57 strain, while C16 manifested a distinct pattern, i.e., a drastically increased ratio of dsRNA1 to dsRNA2 and a lack of DI-dsRNA1 (Fig. 5A). This DI-dsRNA1-free virus was termed RnPV2-DI(Δ/H11002), and the DI-carrying virus was designated RnPV2-DI(Δ/H11001)(Table 1). The elimination of DI-dsRNA1 was corroborated by RT-PCR (see below).

As dsRNA genome segments of partitiviruses are believed to be encapsidated individually (11), the establishment of infection (transfection) requires two types of virions harboring each segment. In accordance with this concept, strain C15 initially acquired all three types of particles, each including dsRNA1, dsRNA2, or DI-dsRNA1 segments, whereas C16 received only dsRNA1 and dsRNA2 particles (Fig. 5B) in amounts sufficient for replication initiation.

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**FIG 1** Properties of the novel mycovirus RnPV2. (A) Colony morphology of virus-infected field strain W57 and virus-cured isogenic strain W57-T25 of *R. necatrix*. Elimination of RnPV2 was carried out by hyphal tipping of strain W57. Strain W57 and virus-cured strain W57-T25 were grown on PDA for 7 days on a benchtop or in the dark at 25°C and photographed. (B) Electron micrograph of purified RnPV2 particles approximately 31 nm in diameter. (C) Protein components of RnPV2 analyzed by SDS-PAGE. Samples were prepared from the purified virus fraction (lane VP) and from a fraction at a position identical to that of virus-free W57-T25 (lane VF) in this and subsequent panels. Lane M, pre-stained protein size standards (Precision Plus Protein Standards; Bio-Rad). (D) Agarose gel electrophoretic analysis of the genomic RNA of RnPV2. Lane M, dsRNA size standards, Mycoreovirus 1 genome segments (32). (E) Genetic organization of RnPV2. Two genomic segments and a DI-RNA-like segment termed dsRNA1 (1,985 nt), dsRNA2 (1,828 nt), and DI-dsRNA1 (1,719 nt), were detected in purified virus preparations. Compared with dsRNA1, DI-dsRNA1 contains a single nucleotide insertion (black arrowhead), a 266-nt deletion in the central portion (shown by a polygonal line), and seven single-nucleotide substitutions (white arrowhead). The 5’-terminal conserved sequence and 3’-terminal poly(A) tail are indicated by gray and black boxes, shown in detail in Fig. 2. The clear boxes indicate ORFs, while the dashed box represents a silent region resulting from the internal stop codon (asterisk in DI-dsRNA1). The core RdRp motif “GDD” (light gray box) is present in this region.
C15 and C16 manifested phenotypes different from that of the virus-free Δdcl-2 mutant that showed a phenotype similar to that of wild-type EP155 (Fig. 5C; see also Fig. 7). RnPV2-DI(−) modified the colony morphology of the Δdcl-2 mutant, causing irregular colony margins, sporadic growth of aerial mycelia, and reduced conidiation at day 12 of culture (Fig. 5C, C16). RnPV2-DI(+) also caused symptoms similar to but milder than those induced by RnPV2-DI(−) (Fig. 5C, C15). RnPV2-DI(+)–infected colonies showed restored growth of aerial hyphae and enhanced asexual sporulation.

RNA silencing targets the partitivirus in C. parasitica regardless of the presence of DI molecules. To determine whether RnPV2 could infect EP155 (RNA silencing–competent wild-type strain), the C15 and C16 strains (Δdcl-2 background) were subjected to hyphal fusion with the EP155 standard strain and the Δdcl-2 mutant in parallel (Fig. 6A). Horizontal viral transmission was analyzed either by agarose gel electrophoresis of dsRNA fractions (data not shown) or by Northern blotting of the ssRNA fractions using dsRNA1- and dsRNA2-specific probes (Fig. 6B). From inspection of Fig. 6B, it was presumed that the movement of RnPV2-DI(+) from the Δdcl-2 mutant to the Δdcl-2 mutant would be verified (lanes 2, 3, and 4), whereas that from the Δdcl-2 mutant to EP155 would not (lanes 9, 10, and 11). However, subsequent RT-PCR confirmed the infection of EP155 by RnPV2-DI(+) at low levels (Fig. 6C). Therefore, DNA fragments of two different sizes (956 and 691 bp) were amplified from dsRNA1 and DI-dsRNA1, respectively, using a primer set targeting a central region of the dsRNA1 including the insertion (1 bp) and deletion (266 bp) regions found in DI-dsRNA1 (Fig. 6C). RnPV2-DI(−) was readily detectable from the Δdcl-2 mutant and EP155 as recipients in a Northern blot assay (Fig. 6B), and this was corroborated by RT-PCR (Fig. 6C, no DI-dsRNA1–derived fragment). The level of RnPV2-DI(−) accumulation in the EP155 strain appeared much lower than that in the Δdcl-2 mutant (Fig. 6B, lanes 5, 6, 7, and 12 versus lanes 13 and 14). This was consistent with RnPV2-DI(+) accumulation. It was concluded that virus accumulation levels were lower in the EP155 strain than in the Δdcl-2 mutant, regardless of the presence of DI molecules. Furthermore, this was consistently observed after repeated subculture (three or six rounds) of the infected fungal colonies (data not shown). Taken together, these findings clearly indicated that the partitivirus was under RNA silencing pressure in C. parasitica.

Note that bands appearing at a migration position similar to that of DI-dsRNA1 transcripts in RnPV2-DI(−) samples (Fig. 6B, shown by asterisks in lanes 5, 6, 7, and 12) were unlikely to be DI segments because no corresponding signals were observed in a Northern analysis using dsRNA fractions (Fig. 5A). In addition, smaller fragments were specifically amplified from RnPV2-DI(+) samples but not from RnPV2-DI(−) samples (Fig. 6C; DI-
dsRNA1). The same notion holds true for Fig. 8A (see lanes T25-Tf18, -Tf19, -Tf22, and -Tf29), illustrating Northern blot assays of RnPV2-DI(-) in the experimental and natural hosts (see below).

**DI-dsRNA1 interferes with RnPV2 replication.** In Fig. 6, the DI nature of DI-dsRNA1 was also demonstrated clearly. The RnPV2 DI-free variant, RnPV2-DI(-), tended to be efficiently multiplied in the Δdel-2 mutant, irrespective of whether it served as a donor or as a recipient, compared to RnPV2-DI(+) (lanes 5,
6, 7, and 12 versus lanes 2, 3, 4, and 9). However, relative accumulation of RnPV2-DI(+) in the Δdcl-2 mutant varied to a great extent; e.g., compare lanes 2 and 4 in Fig. 6B. Furthermore, in EP155, accumulation levels of RnPV2-DI(−) were constantly higher than those of RnPV2-DI(+) in an RNA silencing-competent environment (Fig. 6). These trends were basically maintained until at least six rounds of subculture (data not shown). These findings clearly indicated the DI nature of DI-dsRNA1.

Phenotypic changes caused by DI-carrying and -free RnPV2 infections in C. parasitica. Infection of the two RnPV2 variants in the Δdcl-2 mutant fungal strain caused apparent phenotypic alteration of colony morphology, as shown in Fig. 5C, demonstrating the phenotype of 12-day-old cultures of transfectants. We monitored phenotypic alterations of the recipients after anastomosis at different time points during culturing. RnPV2-DI(+) and RnPV2-DI(−) recipients (7 days old) with the Δdcl-2 background showed a colony morphology characterized by (i) a slightly reduced growth rate accompanied by enhanced orange pigmentation and (ii) a severely reduced growth rate, reduced aerial growth, and enhanced dark brownish pigmentation, respectively (Fig. 7A).
These features were similar to those of C15 and C16 described above, although they fluctuated slightly (Fig. 5C). RnPv2-DI(−) induced much milder symptoms in EP155 recipients than in the ∆dcl-2 mutant, i.e., a slightly reduced growth rate and slightly increased pigmentation (Fig. 7A). Symptoms induced by RnPv2-DI(+) in EP155 recipients were even milder, with hardly any distinction from virus-free EP155 (Fig. 7A). Prolonged culturing accentuated the differences in the aforementioned symptoms among virus-host strain combinations (Fig. 7B). Importantly, the symptoms induced by the two viral strains in the two C. parasitica strains were confirmed further by anastomosing the recipients with virus-free fungal strains (data not shown). These combined observations suggest that reduced RnPv2 accumulation levels by host RNA silencing or DI-dsRNA1 may be associated with milder or little symptom induction.

Effect of DI-dsRNA1 on RnPv2 symptom induction and replication in the natural host, R. necatrix. To elucidate the effects of the DI segment on viral replication, we attempted to transfect the DI-free virus prepared from C16 into the virus-cured original fungus R. necatrix strain, W57-T25. Fungal strains obtained by transformation were examined for dsRNA, and 12 out of 14 were found to be RnPv2-DI(−) positive (data not shown). We selected four transfectant strains designated W57-T25-Tf18, -Tf19, -Tf22, and -Tf29 for subsequent analyses (Table 1).

The RnPv2 accumulation level was drastically elevated by DI-dsRNA1 curing. The dsRNA1 and dsRNA2 transcripts of four DI-free RnPv2 variants were readily detected in a Northern blot assay, but RnPv2 transcripts from the original W57 strain were almost undetectable, except for the DI-dsRNA1 transcript (Fig. 8A, Northern blot assay panels). The authentic dsRNA1 transcript, as well as the dsRNA2 transcript, of DI(+) virus in W57 was detected only when the films were overexposed (data not shown). Accumulation of the dsRNA1 and dsRNA2 segments of RnPv2-DI(−) from sW57-T25-Tf29 was approximately 200 to 400 times higher than that of the original RnPv2 (W57) when measured by a serial-dilution Northern assay (Fig. 8B). The marked increase in RNA was commonly observed in the other three transfection strains (Fig. 8A, compare W57 with T25-Tf18, -Tf19, and -Tf22). Therefore, these results clearly indicated that DI-dsRNA1 caused a significant reduction of helper viral replication.

Unexpectedly, we found different proportions of dsRNA1 and dsRNA2 accumulation among the four transfectants (Fig. 8A, upper panel). In strains W57-T25-Tf18 and -Tf22, dsRNA1 was dominant over dsRNA2, while in W57-T25-Tf19, dsRNA2 was dominant. An almost equal proportion of dsRNA segments was found in strain W57-T25-Tf29. It is believed that no free negative-sense RNA exists in cells infected with a dsRNA virus. Thus, the different ratios of the genome segments are considered to be those of the encapsidated segment.

In contrast to the increase in virus replication caused by the elimination of DI-dsRNA1, the DI-cured virus, RnPv2-DI(−) was unable to induce any overt phenotype alteration of host growth or morphology like DI-dsRNA1-carrying RnPv2-DI(+) (data not shown). RnPv2-DI(−) harboring any proportions of dsRNA did not alter the colony morphology of R. necatrix W57-T25, despite the much greater viral replication than RnPv2-DI(+), suggesting asymptomatic infection of RnPv2 under the restored conditions. Therefore, we concluded that RnPv2 infects R. necatrix asymptotically, regardless of the presence of DI-dsRNA1, as do most partitiviruses.

DISCUSSION

A novel partitivirus isolated from R. necatrix field strain W57 has been thoroughly characterized both biologically and molecularly. This is the second partitivirus to have been isolated/characterized from R. necatrix, and it is thus termed Rosellinia necatrix partitivirus 2 (RnPv2), as proposed by Chiba et al. (23). RnPv2 consists of two dsRNA genome segments; dsRNA1 (1,985 nt) encodes RdRp, and dsRNA2 (1,828 nt) encodes CP, additionally harboring a smaller segment derived from dsRNA1 (DI-dsRNA1, 1,719 nt) with the ability to interfere with the replication of the cognate dsRNA1 (Fig. 1). This is the first report to document the presence of a partitivirus DI-RNA and the first attempt to assess its effects on symptoms and replication levels in natural and experimental fungal hosts. These goals were achieved by using a transfection protocol developed recently for mycoviruses (18, 22, 23, 29, 40) and an RNA silencing-defective ∆dcl-2 mutant fungal strain (26).
It is well known that many plant and animal RNA viruses have subviral RNAs that include DI-RNAs and satellite RNAs (2, 3). DI-RNAs of some plant viruses have been well characterized in view of their biogenesis and effects on helper virus replication and symptom induction. Generally, DI-RNAs interfere with helper viral genome replication, thus alleviating symptom expression. These properties are considered to be attributable to the competitiveness of DI-RNA for host and viral resources over helper virus genomes. Some DI-RNAs have been shown to hinder virally encoded suppressors of RNA silencing by reducing their accumulation or to elevate the accumulation of virus-derived small interfering RNA, leading to enhanced RNA silencing that serves as an antiviral defense mechanism (41). In contrast to information on DI-RNAs of plant RNA viruses, information available for DI-RNAs of mycoviruses is sparse. DI-RNAs have been demonstrated in several hypoviruses with (+) ssRNA genomes, including the prototype hypovirus CHV1 and other hypovirus species (27, 42). Different forms of DI-RNAs with various sizes or heterogeneous DI-RNA populations occur in independent transfecants with in vitro-synthesized transcripts from single full-length cDNA clones of CHV1 (16, 31). CHV3 DI-RNA appears to be maintained stably in field isolate GH2, while it shows considerable sequence divergence from the genomic RNA (42). Whether these hypoviral DI-RNAs affect viral replication and the fungal host phenotype is unknown. DI segments S4ss and S10ss (rearranged dsRNA segments) of a mycoreovirus, Mycoreovirus 1 (MyRV1), have been shown to contribute to altered symptom induction (12, 13, 43). DI segments were also identified in Saccharomyces cerevisiae infected with dsRNA viruses (toviruses, family Toviridae) that were derived from a satellite RNA (called M dsRNA) (44, 45). No report is available for partitivirus DI-RNAs, while some fungal partitiviruses, including Atkinsonella hypoxylon virus (type species of the genus Partitivirus), are known to harbor satellite-like RNAs (46) (summarized in Table S3 in the supplemental material) whose functions are largely unknown. The unavailability of any reverse genetic system for partitiviruses makes it difficult to analyze the effects of such subviral RNAs.

RnP2 DI-dsRNA1 is 266 nt shorter than intact dsRNA1 and could be generated by a dsRNA1 internal deletion event. DI-dsRNA1 would encode a truncated, half-sized form of authentic RdRp (Fig. 1). The truncated form is considered to be defective because of the lack of the central region of RdRp (Fig. 1E). Figures 3, 6, and 8 show that DI-dsRNA1 affects the replication of its parental virus. The ratio of DI-dsRNA1 to intact dsRNA1 was estimated to be 12 to 16:1 in the original isolate, W57 (Fig. 3). A similar ratio was observed in a transfected C. parasitica strain, C15, as assessed by Northern blotting (Fig. 3 and 6). These ratios confirm the nature of DI-dsRNA1 as a DI-RNA and its efficient RdRp template activity, as exemplified by DI-RNAs associated with plant and animal RNA viruses. Depletion of DI-dsRNA1 results in the enhanced replication of helper virus RnP2, as shown by Northern analysis (compare lanes 2 and 9 with lanes 5 and 12, lanes 3 and 4 with lanes 6 and 7, and lanes 10 and 11 with lanes 13 and 14 in Fig. 6B), irrespective of whether the Δdcl-2 mutant or EP155 is used as the host strain. The same trend is evident in the R. necatrix host backgrounds (compare lane W57 with lanes W57-T25-Tf18, -Tf19, -Tf22, and -Tf29 in Fig. 8A). Overall, it is concluded that DI-dsRNA1 acts as a typical DI-RNA to repress its helper virus in both the experimental and original hosts.

RNA silencing was shown to serve as a host defense mechanism against the prototype hypovirus CHV1 and mycoreovirus MyRV1 (26, 47, 48). The elevated RnP2 replication levels, irrespective of whether DI-dsRNA1 is present or absent, in the Δdcl-2 mutant (silencing defective) relative to those in wild-type EP155 (silencing competent) (Fig. 6) clearly indicate that RnP2 is a target of host RNA silencing. This is reminiscent of enhanced replication of MyRV1 in the Δdcl-2 mutant (26) or many plant or insect viruses in RNA silencing-defective hosts (49–53). This is the first example of a member of the Partitiviridae family for which targeting by RNA silencing has been proven. As discussed above, a connection between RNA silencing and DI-RNA has been reported for some plant viruses. A greater degree of reduction of RnP2-DI(+) replication, relative to RnP2-DI(−) replication, was found in EP155 than in the Δdcl-2 mutant (Fig. 6). This observation suggests that in EP155 RnP2-DI(+), replication may be regulated.
by two factors. First, DI-dsRNA1 enhances RNA silencing, leading to a lower level of replication, as observed in (+) ssRNA plant viruses such as tombusviruses (family Tombusviridae) (5, 41). DI-dsRNA1 serves as an RNA synthesis template and possibly as a translation template as well, which may result in competition for host resources in both translation and transcription. Therefore, as the second factor, defective RdRp derived from DI-dsRNA1 may impair functional RdRp by competing for binding with viral RNAs and packaging. In this regard, it will be of interest to examine by Western blotting and/or MALDI-TOF MS analysis whether truncated RdRp protein molecules are present in purified virion fractions from RnPV2(+) -infected mycelia.

RNA silencing was also recently shown to be involved in the generation and maintenance of DI-RNAs of (+) ssRNA viruses (6, 48, 54). In C. parasitica, DCL2 and AGO2, both core players in the RNA silencing pathway (antiviral defense), are required for the production of DI-RNA of CHV1, where they are assumed to provide viral genomic RNA fragments serving as donors and recipients in template switching by RdRp (6, 48). In this study, transfection of the Δdcl-2 mutant of C. parasitica allowed the elimination of DI-dsRNA1. However, for several reasons, it is premature to conclude that RNA silencing mediated the generation of DI-dsRNA1. First, no emergence of DI-RNA was observed in RNA silencing-competent EP155 or W57-T25, even after prolonged maintenance of RnPV1-DI(−) by repeated (10 rounds) subculturing of infected fungal colonies (data not shown). This contrasts with CHV1 DI-RNA, which frequently appeared during the subculturing of infected EP155. Second, repeated horizontal transfer of RnPV1-DI(+) from the Δdcl-2 mutant to the Δdcl-2 mutant failed to eliminate DI-dsRNA1 (data not shown). In contrast, anastomosis of EP155 infected with DI-carrying CHV1 with the Δdcl-2 mutant led to the elimination of DI-RNA, whereas anastomosis with EP155 resulted in stable maintenance of DI-RNA (Fig. 9) when performed side by side in the lateral-transmission experiments shown in Fig. 6A. These findings suggest a fundamental difference in the generation and maintenance of DI-RNA between the two fungal RNA viruses.

Partitiviruses generally show latent infections whether their hosts are plants or fungi (55). This study demonstrated host species-specific symptom induction by RnPV2. The virus induced phenotypic alterations in C. parasitica (Fig. 5 and 7) but not in the natural host, R. necatrix, irrespective of the presence of DI-dsRNA1 (Fig. 1A and data not shown). Given the intracellular life cycle of mycoviruses, it may be considered that RnPV2 has become highly adapted to R. necatrix and developed an innocuous relationship (latent infection) through long-term coexistence. However, its artificial introduction into C. parasitica may perturb such a relationship and induce symptoms. A similar phenomenon was previously observed for another partitivirus from R. necatrix (RnPV1), which enhanced pigmentation in C. parasitica but showed asymptomatic infection in the natural host, R. necatrix (56). The presence of DI-dsRNA1 is associated with reduced symptom severity in either C. parasitica strain (Fig. 7). DI-dsRNA1-free RnPV2 caused a growth rate reduction and enhanced pigmentation in C. parasitica, while DI-dsRNA1-carrying virus induced subtle colony alterations (slightly enhanced pigmentation), which were pronounced at a later culture stage (compare DI(+) with DI(−) in Fig. 7) or in a Δdcl-2 host background (compare EP155 with the Δdcl-2 mutant in Fig. 7). As DI-dsRNA1 and host dcl-2 negatively regulate RnPV2 replication, these symptoms in C. parasitica are likely associated with the enhanced levels of RnPV2 replication.

The introduction of DI-dsRNA1-free RnPV2 back into R. necatrix led to another interesting phenomenon. When evaluated in terms of dsRNA band intensity, the genomic segment dsRNA1- to dsRNA2 ratios of a DI-free partitivirus were shown to vary to a great extent between infected cultures of the natural host, R. necatrix (Fig. 8). For example, in W57-T25-Tf29, dsRNA1 was far more abundant than dsRNA2, as in the C. parasitica Δdcl-2 mutant transfectant (strain C16) (Fig. 5A), while this trend was less pronounced in W57-T25-Tf29. This was in stark contrast to the inverse situation in the other transfectant, W57-T25-Tf19. It is noteworthy that all of these transfectants were derived from a single transfection plate 9 cm in diameter. We monitored the dsRNA profiles for up to 10 subcultures over a period of approximately 4 months. Interestingly, the ratios shown in Fig. 8A fluctuated within some subcultures from the same origin, but on the whole, the marked difference in the ratio was retained. Furthermore, no evident DI-RNA emerged, supporting the notion that DI-dsRNA1 was not generated instantly like CHV1 DI-RNA or that DI-dsRNA1 was not retained in RnPV2 transfectants is surprising. Moreover, the varied proportions of dsRNA found in the original host R. necatrix (Fig. 8A) may represent the segment ratios of virus populations that commence infection at transfection foci that are retained after repeated subculturing.

Using a reproducible transfection technique, DI-RNA was eliminated from the original virus isolate to yield RnPV2-DI(−). An approach similar to that adopted in the present study may allow the elimination of subviral RNAs of other partitiviruses for

FIG 9 Requirement of dcl-2 for sustainable maintenance of CHV1-DI molecules. Northern analysis of CHV1-infected C. parasitica EP155 and Δdcl-2 mutant strains. DI-RNA-carrying CHV1 was laterally transmitted to the recipient EP155 and Δdcl-2 mutant strains. Three independent transmissions were conducted for each strain, and ssRNA fractions obtained from recipient sides were analyzed using a specific cDNA probe hybridizing with the 5'-untranslated region of CHV1. Parental CHV1 donor EP155 and virus-free recipient EP155 and Δdcl-2 mutant strains were analyzed in parallel. The RNAs stained with ethidium bromide are shown as loading controls. DI-RNA present in parental EP155 infected with CHV1 was efficiently transmitted to the recipient EP155 but not to the Δdcl-2 mutant strain, along with CHV1 movement.
which reverse genetic systems are unavailable (see Table S3 in the supplemental material). A model has been proposed to account for DI-dsRNA1 curing as shown in Fig. 5B. As in other partitiviruses, the genomic RNA segments of RnPV2 are believed to be separately encapsidated by CP encoded by dsRNA2 (Fig. 1; see Table S2 in the supplemental material). Thus, the purified virus preparations are mixtures of particles containing individual segments, but the majority contain DI-dsRNA1. It is presumed that transfection efficiency is not high and that infection is established at limited sites in a large transfectant colony (protoplasts were placed at the center of regeneration medium). During regeneration, a population containing both types of particles with dsRNA1 and dsRNA2 establish infection more aggressively than one carrying DI-dsRNA1, whose replication is less efficient.

This transfection technique also allowed us to expand the host range of RnPV2 to C. parasitica, which is placed taxonomically in a different order from the original host, R. necatrix. As discussed above, this finding provided an opportunity to examine whether RNA silencing acts against a partitivirus and to clarify the effects of DI-dsRNA1 of RnPV2 on replication and symptom expression. Furthermore, this study represents an example of a heterologous virus being characterized using an experimental host, C. parasitica. Together with its ease of culture, the availability of genetic manipulation techniques such as transfection and multiple transformation, molecular tools and resources, genome information, and knockout mutants makes C. parasitica amenable to and suitable for studies of virus-host (6, 26, 61–63) and virus-virus interactions (12, 13, 64). Preliminary data have revealed its susceptibility to other heterologous viruses, such as Rosellinia necatrix megabirnavirus 1 (type species of the family Megabirnaviridae) and Rosellinia necatrix victorivirus 1 (a novel victorivirus) (1) (C. parasitica being characterized using an experimental host, C. parasitica). The S4-coded protein is dispensable for viral replication but necessary for efficient vertical transmission and normal symptom induction. Virology 397:399–408.

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