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Exploration of the yadokari/yadonushi nature of YkV3 and RnMBV3 in the original host and a model filamentous fungus

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ABSTRACT

The yadokari/yadonushi nature is a recently discovered virus lifestyle; "yadokari" refers to the ability of capsidless positive-sense (+) RNA viruses (yadokariviruses) to utilize the capsids of phylogenetically distant double-stranded RNA (dsRNA) viruses possibly as the replication site, while "yadonushi" refers to the ability of dsRNA viruses to provide capsids to yadokariviruses. This virus–virus interaction, however, has been only studied with limited pathosystems. Here, we established a new study model with a capsidless (+)RNA yadokarivirus YkV3 (family *Yadokariviridae*) and its capsid donor RnMBV3 (family *Megabirnaviridae*) in the original host fungus *Rosellinia necatrix* and a model filamentous fungal host *Cryphonectria parasitica*. YkV3 has a simple genome structure with one open reading frame of 4305 nucleotides encoding a single polyprotein with an RNA-dependent RNA polymerase and a 2A-like self-cleavage peptide domain. Reverse genetics of YkV3 in *R. necatrix* showed that YkV3 tolerates a nucleotide substitution in the extreme 5′-terminus. The insertion of two termination codons immediately downstream of the 2A-like cleavage site abolished YkV3 viability, suggesting the importance of the C-terminal portion of the polyprotein of unknown function. Transfection of RnMBV3 and YkV3 into an RNA silencing-deficient mutant Δ*dcl2* of *C. parasitica* showed the replication competency of both viruses. Comparison between the wild-type and Δ*dcl2* strains of *C. parasitica* in virus accumulation suggested that RnMBV3 and YkV3 are susceptible to RNA silencing in *C. parasitica*. Taken together, we have established a platform to further explore the yadokari/yadonushi nature using genetically manipulable host fungal and virus strains.

1. Introduction

The yadokari/yadonushi nature is a recently discovered virus lifestyle, where a capsidless positive-sense (+) single-stranded (ss) RNA yadokarivirus diverts the capsid of a partner double-stranded (ds) RNA virus to hetero-encapsidate its RNA and RNA-dependent RNA polymerase (RdRP) (Das et al., 2021; Das and Suzuki, 2021; Hisano et al., 2018; Zhang et al., 2016). The Japanese word "yadokari" literally means "room borrower or hermit crab," while "yadonushi" means "room owner." (Sato et al., 2023; Zhang et al., 2016) Thus, the yadokari nature refers to the ability of a yadokarivirus to highjack the capsid of a distinct dsRNA virus, while the yadonushi nature is the ability of the partner to supply its own capsid to help the corresponding yadokarivirus replicate. Thus far, these peculiar viruses have been discovered from diverse filamentous fungi (Sato et al., 2023). Taxonomically, yadokariviruses belong to the family *Yadokariviridae* in the order *Yadokarivirales* within the phylum *Pisuviricota* (Sato et al., 2023; Walker et al., 2022). The family *Yadokariviridae* is further divided into the genera *Alphayadokarivirus* or *Betayadokarivirus*, according to the phylogeny based on the RdRP amino acid sequence. Each yadokarivirus appears to have a species-specific dsRNA virus partner among the diverse unisegmented and bisegmented members of the order *Ghabrivirales* (Jia et al., 2022; Sato et al., 2022a). In addition to yadokaviruses in filamentous fungi, an umbra-like virus in a plant is suggested to be hetero-encapsidated by a dsRNA virus in the order *Ghabrivirales*; these viruses may also have the yadokari/yadonushi nature (Sa Antunes et al., 2016, 2020).

Rosellinia necatrix is an ascomycetous plant pathogen to many annual and perennial crops (Pliego et al., 2012). The above-mentioned new virus lifestyle was discovered in *R. necatrix* for the first time and since then has been intensively studied in this fungus where yadokari virus (YkV) 1–4 have been identified and characterized (Arjona-Lopez et al., 2018; Zhang et al., 2016). YkV1 belongs to the genus *Alphayadokarivirus*,

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while YkV2 to YkV4 belong to the genus *Betayadokarivirus* (Sato et al., 2023). Interesting genomic features of these yadokariviruses include the heterogeneity of the extreme termini and different numbers of open reading frames (ORFs). Specifically, YkV1, YkV2, and YkV3 possess single ORFs encoding a polyprotein likely processed by a 2A-like self-cleavage peptide motif (–GDVExNPG↓P–) that triggers ribosome skipping (Luke et al., 2008) and production of an RdRP and a protein of unknown function. To date, this polyprotein processing event has been substantiated only for YkV1 (Das et al., 2021). By contrast, two distinct strains (a and b) of YkV4 each have two ORFs encoding an RdRP and a protein corresponding to C-terminal portion of the polyprotein of the former yadokariviruses. The heterogenous nucleotide at the 5′-terminal genome end has been reported for several betayadokariviruses (Sato et al., 2023). YkV2, YkV3, and YkV4b have either a C or a U residue, while YkV4a has either a C or an A residue at the extreme 5′-terminus (Arjona-Lopez et al., 2018). Whether both of the residues are capable of initiating replication is not known.

There are complex interactions among yadokariviruses, dsRNA virus partners, and host fungi (Sato et al., 2022a). For example, YkV1 appears to trans-enhance the accumulation of its partner yadonushi virus 1 (YnV1) (of the proposed family "Yadonushiviridae"), possibly leading to reduced growth of the host fungus (Zhang et al., 2016). YkV4 interferes with the replication of its partner dsRNA virus, Rosellinia necatrix megatotivirus 1a (RnMTV1a) (of the proposed family "Megatotiviridae"), which alone causes reduced growth of the host fungus, and restores the fungal growth defect (Sato et al., 2022a). YkV3 has neither a positive nor a negative impact on the partner dsRNA virus, Rosellinia necatrix megabirnavirus 3 (RnMBV3) (of the family *Megabirnaviridae*), or the host fungus (Sato et al., 2022a). One of the interesting unexplored aspects of these pathosystems is how host genes can be involved in the yadokari/yadonushi interaction. For example, it is unknown whether antiviral RNA silencing, the primary antiviral defense in fungi, functions against yadokariviruses and their partners. In this regard, the chestnut blight fungus, *Cryphonectria parasitica*, is suitable for addressing such a question, because there are many biological and molecular tools available for this fungus (Eusebio-Cope et al., 2015). Furthermore, this fungus is known to support viruses from many other different host fungi including those from *R. necatrix* (Chiba et al., 2013, 2016; Salaipeth et al., 2014). However, no yadokariviruses and capsid donor dsRNA viruses are known to infect *C. parasitica*.

In the current study, we explore the yadokari/yadonushi nature of the betayadokarivirus YkV3 and its partner RnMBV3 in the original host *R. necatrix* and the experimental host *C. parasitica*. In *R. necatrix*, we established reverse genetics of YkV3 to show the flexibility in the 5′-terminal nucleotide and the requirement of the C-terminal part of polyprotein with unknown function for YkV3 replication. Furthermore, we successfully transfected *C. parasitica* with YkV3 and RnMBV3, which allowed us to investigate the effects of host RNA silencing against these viruses. Collectively, we show the advantage of the YkV3-RnMBV3-*R. necatrix*/*C. parasitica* system to study the yadokari/yadonushi nature using a genetically manipulable virus and host.

2. Materials and methods

2.1. Fungal strains and growth condition

Rosellinia necatrix strain Rn454 as a source of YkV3 and RnMBV3 (Arjona-Lopez et al., 2018) and the virus-free *R. necatrix* strain W97 (Shimizu et al., 2018) were generous gifts from Dr. Carlos José López-Herrera and Satoko Kanematsu, respectively. The transfectant of W97 with RnMBV3 was generated previously (Sato et al., 2022a). T-YkV3, the transformant of W97 with an infectious complementary DNA (cDNA) clone of YkV3 whose 5′-terminus starts with U was also generated previously (Sato et al., 2022a). The other transformants of W97 (T-YkV3$_{U1C}$ and T-YkV3$_{2Astop}$) were generated in this study (see below). *Cryphonectria parasitica* strain EP155 and its derivative Δ*dcl2*, an RNA silencing–deficient mutant (Segers et al., 2007), were provided by Dr. Donald L. Nuss. The fungal strains were cultured on potato dextrose agar (PDA, Difco) at room temperature (around 25 °C) in the dark for *R. necatrix* or in natural daylight for *C. parasitica*.

2.2. Vector construction

Full-length cDNAs to a variant and a mutant of YkV3, namely YkV3$_{U1C}$ and YkV3$_{2Astop}$ were commercially synthesized (GENEWIZ, Inc.). YkV3$_{U1C}$ had a C residue at the extreme 5′-terminus instead of U, while YkV3$_{2Astop}$ lacked the ability to translate the C-terminal coding region after the putative 2A-like cleavage site because of the substitution of two stop triplets (UAA and UGA) for the two codons behind the cleavage site (Fig. 1A). Translation would stop after addition of a glycine at position 1021 on the YkV3 ORF1 product (GenBank accession: BBB86810.2) (Fig. 1A). These cDNAs were inserted into the dual ribozyme cassette of the fungal expression vector pCPXHY3 (Guo et al., 2009) with the hammerhead ribozyme (HHRz) and the hepatitis delta virus ribozyme (HDVRz) at the 5′- and 3′-terminus, respectively (Fig. 1B), as described previously (Sato et al., 2022a; Zhang et al., 2016).

2.3. Transformation of R. necatrix

Protoplasts of *R. necatrix* strain W97 were prepared as described previously(Kanematsu et al., 2004). These protoplasts were transformed with mutant YkV3 cDNA plasmid as described previously (Sato et al., 2022a). The transformants with YkV3$_{U1C}$ or YkV3$_{2Astop}$ cDNAs were termed T-YkV3$_{U1C}$ or T-YkV3$_{2Astop}$ respectively. Replication competency of these mutants was tested after the inoculation of RnMBV3 by the hyphal fusion method as described previously (Fig. 1C) (Sato et al., 2022a).

2.4. Transfection of C. parasitica

Transfection of *C. parasitica* with virus particles is a multi-step procedure: preparation of fungal protoplasts, isolation of virus particle fractions, their introduction into protoplasts, and protoplast regeneration. Protoplasts of *C. parasitica* strain Δ*dcl2* on the EP155 background were prepared as described previously (Churchill et al., 1990) with modifications. For fungal preculture, six 5 mm^3 mycelial plugs were placed in separated positions on PDA and cultured for 2 days at room temperature. The six mycelial circles on PDA were hollowed out with a sterile blade, transferred to 100 mL potato dextrose broth (PDB, Difco) in a 200 mL flask, and incubated at room temperature in the dark for 2 days. The newly extended hyphae from the PDA plugs were scraped off with a sterile spatula or blade, transferred to 400 mL of PDB in a 1-L flask, and incubated at room temperature in the dark for 2 days. The mycelia were harvested with sterile Miracloth and washed with 0.6 M $MgSO_4$. The washed mycelia were transferred to a 100 mL flask and mixed with 25 mL of filter-sterilized digestive enzyme mix [1% (v/v) β-glucuronidase (Sigma-Aldrich, #G1512), 0.67% (w/v) lysing enzyme (Sigma-Aldrich, #L1412), and 1% (w/v) bovine serum albumin] in the osmotic medium [1.2 M $MgSO_4$ and 10 mM NaH_2PO_4, adjusted to pH 5.8 with a certain volume of 1 M Na_2HPO_4]. The digestion was performed at 28 °C at 80 rpm for 3 h. The digestive mixture was transferred to a 50 mL tube, overlayed with 20 mL of trapping buffer (0.4 M sorbitol in 100 mM Tris–HCl pH 7.0), and centrifuged at ~2000 *g* for 20 min at 4 °C in a swing rotor. The middle layer was transferred to a new 50 mL tube, mixed with the twice volume of 1 M sorbitol, and centrifuged at ~2000 *g* for 5 min at 4 °C. The pelleted cells were resuspended in 10 mL of STC (1 M sorbitol, 100 mM Tris–HCl pH 8.0, and 100 mM $CaCl_2$) and centrifuged at ~2000 *g* for 5 min at 4 °C. The pelleted cells were resuspended in a moderate volume of STC to 1–2 × 10^7 cells/mL on ice. The cells were mixed with a quarter volume of PTC [40% (w/v) polyethylene glycol (PEG, Mw 4000) in 100 mM Tris–HCl pH 8.0 and 100 mM $CaCl_2$] and 1% (v/v, final) dimethyl sulfoxide to store at −80 °C.

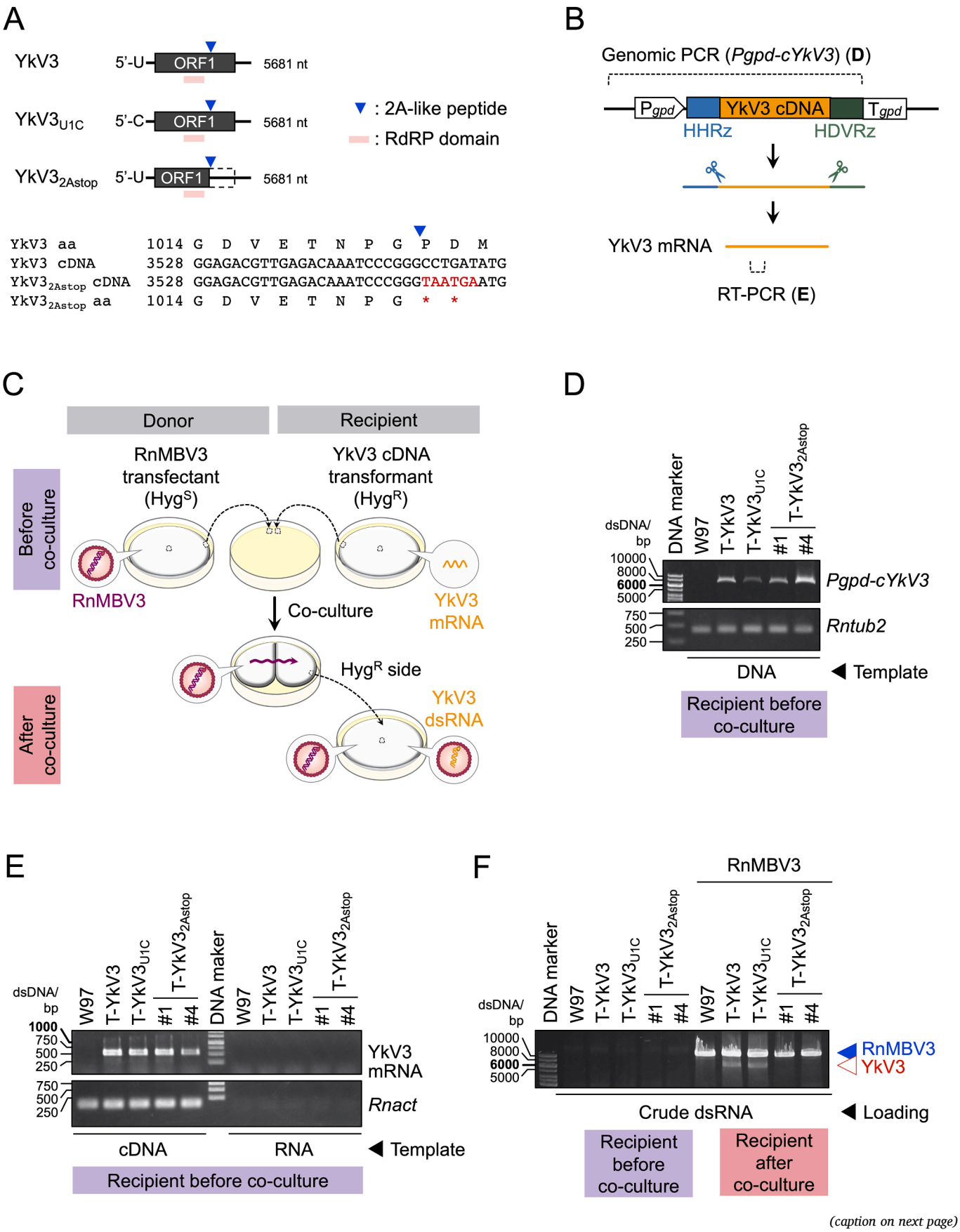

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Fig. 1. Reverse genetics of a betayadokarivirus, YkV3. (**A**) Schematic representation of genomic RNA of YkV3 and its variant or mutant, YkV3$_{U1C}$ and YkV3$_{2Astop}$. The nucleotide sequence alignment with translated amino acid sequences shows the mutation site of YkV3$_{2Astop}$ compared with wild-type YkV3. (**B**) Schematic diagram of the infectious cDNA clone of the YkV3 variants introduced into *Rosellinia necatrix* genome. P*gpd* and T*gpd* denote the promoter and terminator of glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) of *Cryphonectria parasitica*, respectively. HHRz and HDVRz refer to the hammerhead ribozyme and hepatitis delta virus ribozyme, respectively. The dotted lines indicate regions amplified by host genomic PCR or RT-PCR in (**D**) and (**E**). (**C**) Schematic representation for the method to inoculate RnMBV3 to the transformants. The RnMBV3 donor strain [*R. necatrix* W97 transfected with RnMBV3, hygromycin susceptible (Hyg^S)] was co-cultured with respective recipient strains [YkV3 cDNA transformants (each one trasnformant of T-YkV3 and T-YkV$_{U1C}$, and two independent transformants of T-YkV3$_{2Astop}$), hygromycin resistant (Hyg^R)]. RnMBV3 can be horizontally transferred to the recipient side via hyphal fusion of the co-cultured strains. The recipient side after co-culture was selected based on hygromycin resistance. (**D**) Host genomic PCR to confirm integration of the full-length YkV3 cDNA expression cassette (*Pgpd-cYkV3*). The β-tubulin gene of *R. necatrix* (*Rntub2*) was detected as an internal control. (**E**) RT-PCR to confirm the expression of YkV3 transgenes. The actin gene of *R. necatrix* (*Rnact*) was detected as an internal control. The non-reverse-transcribed RNA template was used to check for the absence of contaminated host genomic DNA. (**F**) The electrophoretic profile of total dsRNA extracted from the transformants with YkV3 variants before and after receiving its partner dsRNA virus RnMBV3. Virus-free W97 and W97 transfected with RnMBV3 were examined in parallel as negative controls for RnMBV3/YkV3 and YkV3, respectively.

The transfection of *C. parasitica* strain Δ*dcl2* (hygromycin resistant) with RnMBV3 and YkV3 was performed as illustrated in Fig. S1A. The inoculum crude virus particle (VP) fraction was obtained from *R. necatrix* strain W97 that had been infected with both RnMBV3 and YkV3 previously (Sato et al., 2022a). The protoplasts of *C. parasitica* strain Δ*dcl2* were transfected with the crude VPs of RnMBV3 and YkV3 according to the PEG-mediated transformation method (Churchill et al., 1990), as Hillman et al. (Hillman et al., 2004) applied to virion transfection. Briefly, 100 μL of the frozen-thawed protoplasts were mixed with 20 μL of the crude VP fraction and incubated on ice for 30 min. The protoplasts and VP suspension were combined with 1 mL of PTC, mixed by inversion, and incubated at room temperature for 25 min. The protoplasts were pelleted by centrifugation at 8000 × *g* for 5 min at 4 °C. The pellets were roughly washed with 500 μL of STC and again centrifuged at 6000 × *g* for 2 min at 4 °C. The pellets were resuspended in 1 mL of STC on ice. The protoplast suspension was separately dropped off into the center of four petri dishes (9 cm in diameter) and mixed with 20 mL of the regeneration medium [0.1% (w/v) yeast extract, 0.1% (w/v) casein hydrolysate, 1 M sucrose, and 1.6% (w/v) agar]. The fungal colonies were paired in the medium by co-culturing for 1 week at room temperature in natural daylight. The mycelial plugs were picked from distant positions and transferred to PDA containing 50 μg/mL hygromycin B. After selection with hygromycin, the independent colonies were maintained as independent transfectants on PDA containing no antibiotics.

2.5. DNA extraction and genotyping

DNA was extracted from *R. necatrix* as described previously (Sato et al., 2022a). Full-length YkV3 cDNA transgene insertion was checked by polymerase chain reaction (PCR) with PrimeSTAR GXL DNA Polymerase (Takara Bio Inc.) in a 10 μL reaction volume with the *R. necatrix* DNA template and primers listed in Table S1. PCR products were electrophoresed in a 1% agarose gel in 0.5 × TAE and stained with ethidium bromide. As a DNA marker, GeneRuler 1 kb DNA Ladder (Thermo Scientific) was used for all the experiments including the following reverse-transcription PCR (RT-PCR) and dsRNA visualization.

2.6. RNA extraction, RT-PCR, and northern blotting

RNA molecules (ssRNA and dsRNA) were extracted from *R. necatrix* and *C. parasitica* as described previously (Sato et al., 2022a). Briefly, ssRNA-enriched total RNA was prepared via lithium chloride precipitation of RNA from total nucleic acids. Alternatively, total RNA was purified via treatment with DNase for the total nucleic acids. Crude dsRNA was extracted by the cellulose binding affinity method without following treatment with DNase and S1 nuclease.

RT-PCR with purified RNA was performed in two steps with M-MLV Reverse Transcriptase (Invitrogen) and Quick Taq HS DyeMix (TOYOBO) (Fig. 1E) or in one step with PrimeScript One Step RT-PCR Kit, Ver.2 (Dye Plus, TaKaRa Bio) (Figs. 2A, 3B, and 4) as described previously(Sato et al., 2022a), with the primers listed in Table S1. The mycelial direct multiplex RT-PCR was performed as described previously (Sato et al., 2020), with the mixed RnMBV3 and YkV3 primer sets listed in Table S1. The terminal sequence of YkV3 transfected into *C. parasitica* was determined by the rapid amplification of cDNA ends (RACE) method as described previously (Lin et al., 2012), with a viral specific primer in Table S2. The consensus sequence was determined by analysis of more than 10 clones.

Northern blot was performed in an agarose–MOPS–formaldehyde denaturing gel using ssRNA-enriched total RNA as described previously (Sato et al., 2022b). Viral cDNA probes for the northern blot were generated as described previously (Sato et al., 2022a), with primers listed in Table S1. The band intensity on the northern blot image was quantified with ImageJ version 1.53t according to the provider's instructions (https://imagej.nih.gov/nih-image/manual/tech.html). Statistical analysis was performed with the ggsignif version 0.6.4 and multcomp version 1.4.23 packages in R version 4.1.1 (https://cran.r-project.org/, https://cran.r-project.org/web/packages/ggsignif, https://cran.r-project.org/web/packages/multcomp).

3. Results

3.1. Reverse genetics of a representative betayadokarivirus, YkV3

Prior to this study, reverse genetics of a yadokarivirus had only been conducted with an alphayadokarivirus, namely YkV1 (Das et al., 2021). Here we performed reverse genetics of a betayadokarivirus, YkV3, to address two key questions for betayadokariviruses. The first regards the sequence heterogeneity at the extreme 5′-terminus observed in several betyadokariviruses (*e.g.*, C or U in YkV3). We tested whether full-length cDNA with either heterogenous nucleotide could launch virus replication. Second, we examined whether the C-terminal portion of putative YkV3 polyprotein after 2A-like peptide was required for the replication as in the case of YkV1. Note that this C-terminal portion shares a low level (20%) of amino acid sequence identity (Fig. S2). To address these questions, we prepared two YkV3 cDNA mutants: a 5′-end substitution mutant (YkV3$_{U1C}$), which has a C in place of U at the 5′ end, and a 3′ proximal coding domain deficient mutant (YkV3$_{2Astop}$), which has a double termination codon UAAUGA immediately downstream of codon GGG$_{3551}$/ for the 2A cleavage site Gly/Pro (G$_{1021}$↓P$_{1022}$) (Fig. 1A). Both YkV3 cDNA mutants were cloned into the fungal expression vector pCPXHY3 (Fig. 1B). We introduced these constructs into *R. necatrix* strain W97 to check the production of YkV3 replicative form dsRNA after receiving capsid donor RnMBV3 by the co-culture with an RnMBV3 transfectant (Fig. 1C). We named the transfectants with YkV3 with 5′-U (here described as wild-type YkV3) (Sato et al., 2022a), YkV3$_{U1C}$, and YkV3$_{2Astop}$ as T-YkV3, T-YkV3$_{U1C}$, or T-YkV3$_{2Astop}$ respectively. Before the RnMBV3 inoculation, we confirmed the integration of the full-length transgene and its transcription in the transformants (Fig. 1D and E). After the inoculation of RnMBV3, YkV3$_{U1C}$ as well as wild-type YkV3 produced the replicative form of dsRNA but YkV3$_{2Astop}$ did not (Fig. 1F). This suggests that YkV3 can initiate replication with either C or U at the 5′-terminus, but it requires the protein product encoded by the 3′

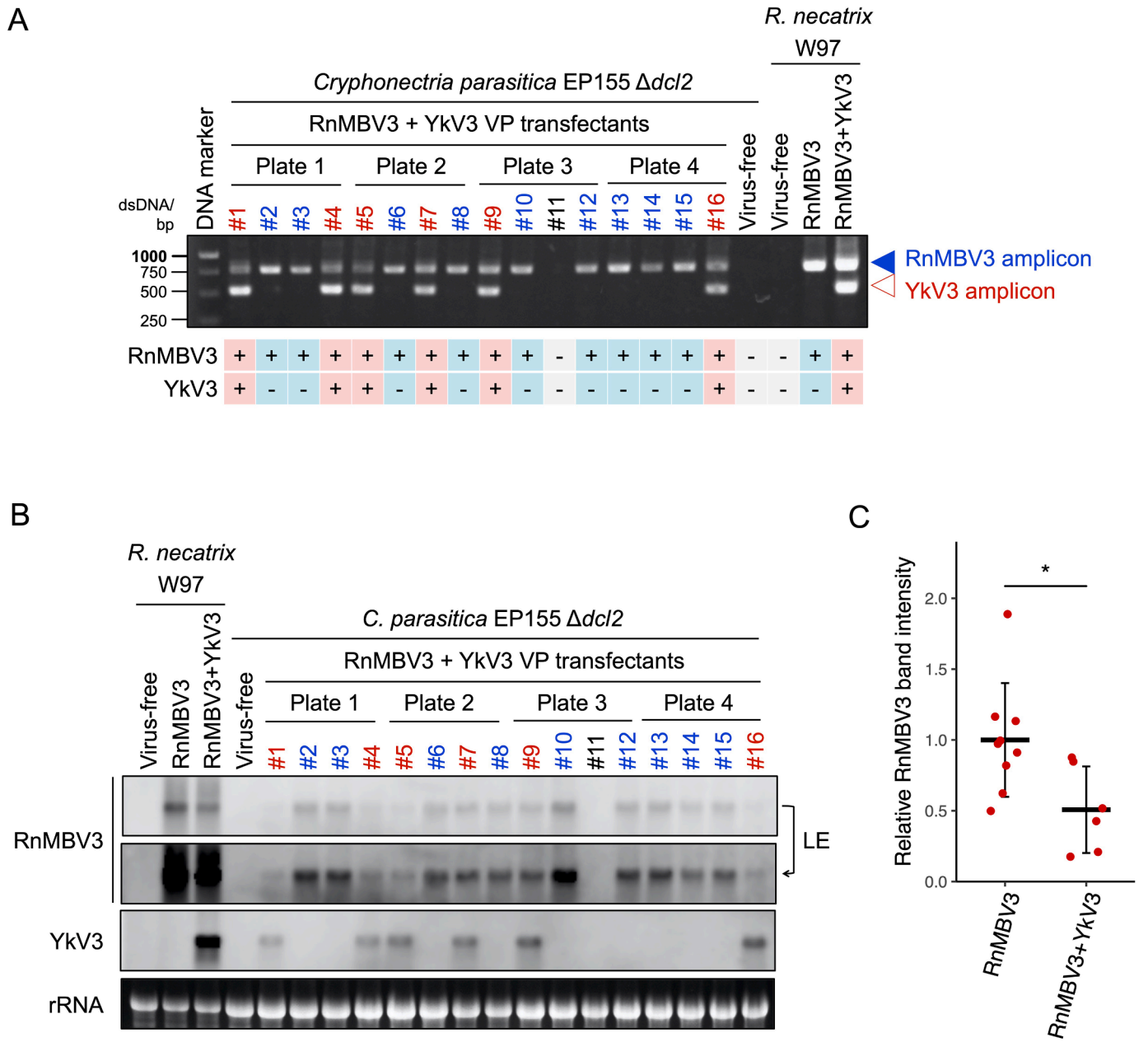

Fig. 2. Replication of YkV3/RnMBV3 in a model filamentous fungal host, *Cryphonectria parasitica*. An RNA silencing–deficient strain (Δ*dcl2*) of *C. parasitica* EP155 was transfected with mixed virions of RnMBV3 and YkV3 (Fig. S1). The presence or absence of each virus was determined in 16 subcultures of the transfectants from four regeneration plates. Virus-free *C. parasitica* Δ*dcl2* and *Rosellinia necatrix* W97 were used as negative controls for viral detection. *Rosellinia necatrix* infected by RnMBV3 alone or together with YkV3 was used as positive controls. (**A**) Detection of RnMBV3 and YkV3 by multiplex colony RT-PCR. RnMBV3 and YkV3 were distinguishably detected with longer or shorter amplicons, respectively. (**B**) Detection of RnMBV3 and YkV3 by northern blot. RnMBV3 and YkV3 were separately detected with cDNA probes for each virus. The RNA loading amount in each lane was 10 or 4.8 μg for the detection of RnMBV3 and YkV3, respectively. "LE" stands for a longer exposure for chemiluminescence detection. Fungal ribosomal RNA (rRNA) stained with ethidium bromide is shown as RNA loading control (the gel for YkV3 detection is representatively shown). (**C**) Comparison of relative RnMBV3 band intensity between the transfectants infected with only RnMBV3 or co-infected with RnMBV3 and YkV3. The relative band intensity was quantified based on the northern blot image with longer exposure in (**B**). The relative band intensity was calculated by dividing the raw value by the mean value of the RnMBV3 single transfectants. Each red plot represents the relative band intensity of each transfectant. The crossbar indicates the mean value of each group. The error bar indicates the standard deviation. The asterisk indicates a significant difference detected by the *t*-test ($p < 0.05$, $n = 6$ or 9).

proximal portion for replication.

3.2. Replication competency of YkV3 in a model filamentous fungal host, *C. parasitica*

To expand the host system for investigation of the yadokari/yadonushi nature, we attempted to introduce RnMBV3 and YkV3 into an artificial host, namely *C. parasitica*. As a host fungal strain, we selected the RNA silencing–deficient mutant Δ*dcl2* (EP155 background) (Segers et al., 2007) because a low replication level of YkV3/RnMBV3 could be expected in wild-type *C. parasitica* as is observed for other viruses from *R. necatrix* (Chiba et al., 2013). We infected protoplasts of strain Δ*dcl2* with a VP fraction purified from *R. necatrix* W97 co-infected with RnMBV3 and YkV3 (Sato et al., 2022a), as illustrated in Fig. S1A. The transfected protoplasts were regenerated in four independent plates. We took four distal mycelial plugs from each regeneration plate and

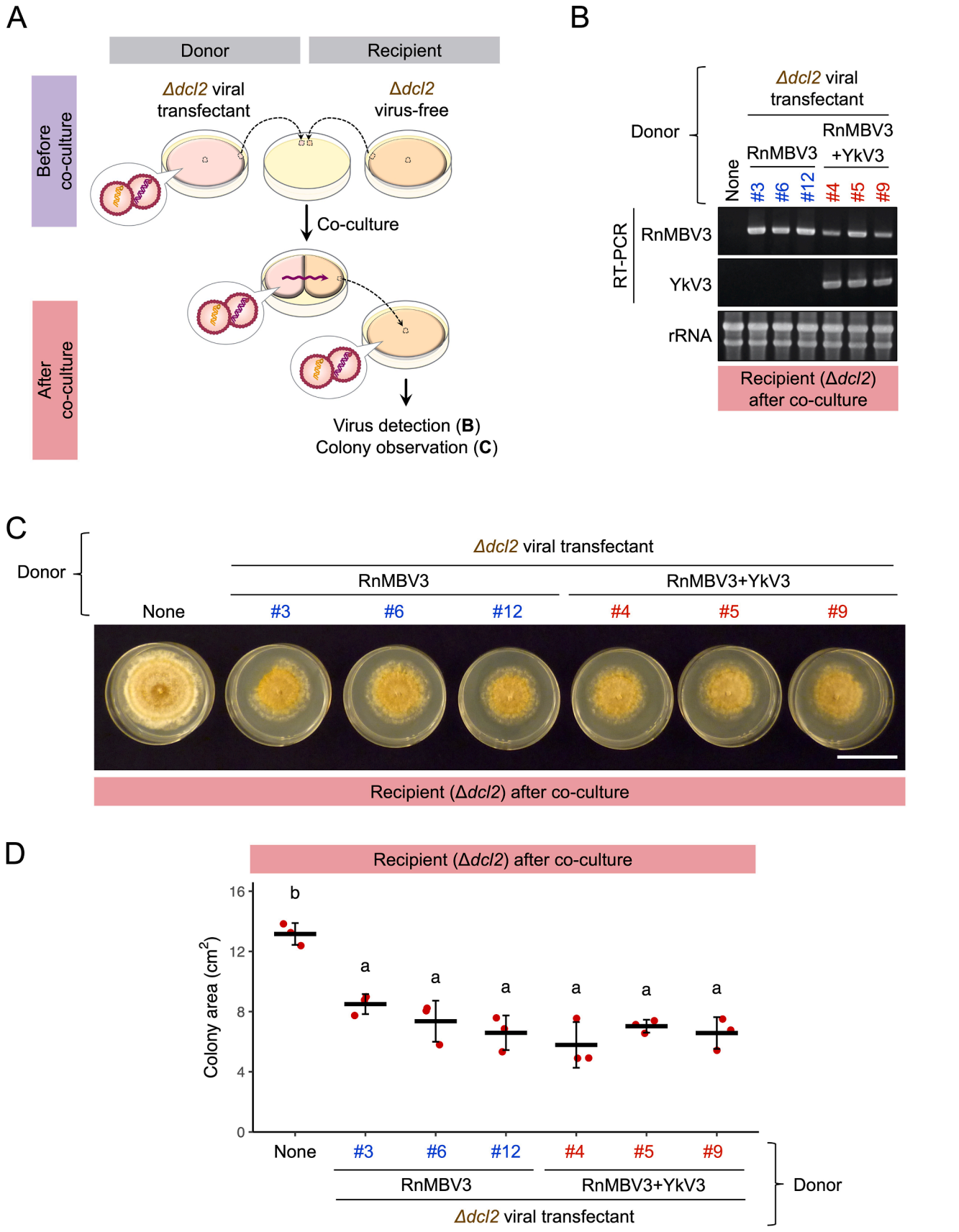

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Fig. 3. Effect of RnMBV3 and YkV3 on *in vitro* growth of *Cryphonectria parasitica* Δ*dcl2*. (**A**) Schematic representation of the method for re-inoculation of the Δ*dcl2* strain with viruses from the transfectants. The viral donor strains (Δ*dcl2* transfected with RnMBV3 alone or with RnMBV3 and YkV3 together, Figs. S1 and 2) were co-cultured on PDA with the virus recipient (virus-free Δ*dcl2*) for around 1 week. The recipient side after the co-culture was transferred to a new PDA to check the viral presence (**B**) and colony morphology (**C**). (**B**) Virus detection by RT-PCR with total RNA purified from the recipient Δ*dcl2* strain after the co-culture. The upper two panels show RT-PCR products of the respective viruses. The lower panel shows fungal ribosomal RNA (rRNA) in the template total RNA stained with ethidium bromide. For the rRNA visualization, 5 μg total RNA was loaded. (**C**) Colony morphology of the recipient Δ*dcl2* received the viruses. A mycelial plug (1 mm^3) was transferred to PDA in 5.5 cm diameter, cultured for five days, and photographed. The white bar indicates 3 cm. (**D**) Measurement of colony area of the virus recipient Δ*dcl2*. The area of fungal colonies (C) was measured using the wand tool of ImageJ version 1.53t The values of three plates were measured for each recipient and shown with the red points. The mean values plus/minus standard deviations were indicated with black crossbars. Different alphabets indicate statistical differences by Tukey test ($n = 3$, $p < 0.05$).

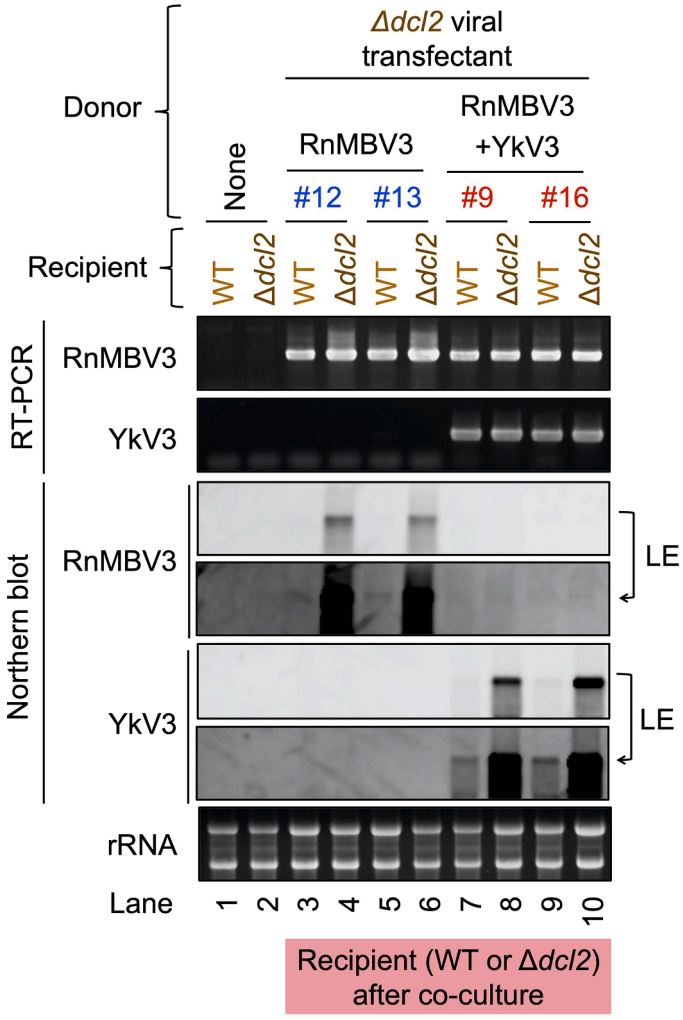

Fig. 4. Impact of *dcl2* on RnMBV3 and YkV3 accumulation in *Cryphonectria parasitica*. The wild-type (WT) and Δ*dcl2* strains (EP155 background) were inoculated with RnMBV3 alone or with RnMBV3 and YkV3 together in the same system as Fig. 3A. RNA was extracted from the WT and Δ*dcl2* strain after the co-culture with the indicated viral donors and used for RT-PCR (upper panels) and northern blotting (middle panels). For northern blotting, 2 μg of the total RNA was loaded into each well. "LE" stands for a longer exposure for chemiluminescence detection. The bottom panel shows fungal rRNA stained with ethidium bromide as a total RNA loading control.

maintained them on PDA as independent subcultures. We first investigated the presence or absence of RnMBV3 and YkV3 by direct multiplex RT-PCR of mycelia from 16 subcultures obtained from four regeneration plates (Fig. 2A). The multiplex RT-PCR showed two types of infections: single infection by RnMBV3 (nine subcultures) and double infection by YkV3 and RnMBV3 (six subcultures). We also observed this pattern when we transfected YkV1/YnV1 particles into the protoplasts of *R. necatrix* (Zhang et al., 2016). Next, we analyzed the accumulation level of RnMBV3 and YkV3 in the Δ*dcl2* strain by northern blotting (Fig. 2B). The accumulation level of both viruses was generally lower in the Δ*dcl2* strain than the original host *R. necatrix* (Fig. 2B), while both northern blots clearly showed replication of both RnMBV3 and YkV3 in *C. parasitica* Δ*dcl2*. The accumulation level of RnMBV3 in the Δ*dcl2* strain was uneven among the transfectants and tended to be higher in the singly infected subcultures than the doubly infected subcultures with YkV3 (Fig. 2B and C).

To confirm the full-length YkV3 introduced in the *C. parasitica* Δ*dcl2* transfectants, we conducted RT-PCR with a series of six primer sets in several transfectants (RnMBV3/YkV3 co-transfectants #4, #5, #9, and #16). These primer sets amplified different 1 kb regions covering the entire genome with a short overlap between them (Table S2). Electrophoresis in a 1% agarose gel showed no differences in the amplified fragment length or presence/absence, except for the most 3′-terminal region of YkV3 (data not shown). Therefore, we identified the 3′-terminal sequence of YkV3 in the transfectants by RACE. This assay revealed that YkV3 in the transfectants lacked 116 nucleotides (nucleotides 5553–5668 in the reference genome of isolate Rn454, accession number LC333757.2) immediately before the poly(A) tail in the 3′-untranslated region (UTR) (Fig. S3). It remains unknown how this deletion occurred, or what its biological significance was.

3.3. Effect of RnMBV3 and YkV3 on in vitro growth of C. parasitica Δdcl2

C. parasitica Δ*dcl2* infected with RnMBV3 alone or together with YkV3 showed an abnormal phenotype on PDA distinct from the original virus-free Δ*dcl2* strain and a virus-free transfectant subculture (#11) (Fig. S1B). To confirm whether this abnormal phenotype was caused by viral infection, we re-inoculated these viruses into the virus-free Δ*dcl2* strain by co-culturing with each transfectant infected with RnMBV3 alone (subcultures #3, #6, and #12) or plus YkV3 (subcultures #4, #5, and #9) (Fig. 3A). After the co-culture, we used RT-PCR to confirm the presence of the viruses at the recipient side (Fig. 3B). The virus-transferred recipient side showed the abnormal colony morphology—that is, delayed growth, biomass reduction, and darker pigmentation (Figs. 3C and 3D)—as observed in the original transfectants (Fig. S1B). The phenotype seemed not to differ by the presence or absence of YkV3 (Figs. 3C and S1B). These results suggest that RnMBV3 causes severe symptoms in *C. parasitica* Δ*dcl2*, to which YkV3 makes little or no contribution. This contrasts with the case in *R. necatrix* W97 in which neither RnMBV3 alone nor RnMBV3 together with YkV3 alters the host colony phenotype in a similar experimental condition (Sato et al., 2022a).

3.4. Effects of RNA silencing in C. parasitica on RnMBV3 and YkV3 accumulation

Lastly, we tested whether RnMBV3 and YkV3 could replicate in wild-type *C. parasitica* EP155 that has a functional antiviral RNA silencing machinery (Segers et al., 2007). We inoculated *C. parasitica* EP155 wild-type and Δ*dcl2* as virus recipients by co-culturing with each virus donor similarly as shown in Fig. 3A. As virus donor strains, we used two subcultures each of RnMBV3 single transfectants (subcultures #12 and

#13) and RnMBV3+YkV3 double infectants (subcultures #9 and #16). We confirmed viral horizontal transmission to the recipient sides by using RT-PCR (Fig. 4 upper panels). Then, we analyzed the accumulation level of viral RNA by northern blotting (Fig. 4 lower panels). Comparison of the RnMBV3 signal between the wild-type and Δ*dcl2* strains in the RnMBV3 single inoculation showed that the accumulation level of RnMBV3 was much lower in wild-type strain than in the Δ*dcl2* strain (Fig. 4, northern blot for RnMBV3, lanes 3–6), suggesting that RnMBV3 was targeted by RNA silencing. This trend was not confirmed in the co-infection with RnMBV3 and YkV3, because RnMBV3 accumulation was too low to detect even in Δ*dcl2* by northern blotting (Fig. 4, northern blot for RnMBV3, lanes 7–10). We clearly detected YkV3 northern signal in these co-infected subcultures, where the accumulation level of YkV3 was also much lower in the wild-type EP155 strain than the Δ*dcl2* strain (Fig. 4, northern blot for YkV3, lanes 7–10).

4. Discussion

The yadokari/yadonushi nature has been extensively studied by using the phytopathogenic filamentous fungus *R. necatrix*, from which the first yadokarivirus was discovered, despite the fact that many filamentous fungi can host yadokariviruses. Among the yadokariviruses, an alphayadokarivirus, namely YkV1, has been studied most intensively (Das et al., 2021; Hisano et al., 2018; Zhang et al., 2016), while betayadokariviruses have been less investigated. We established reverse genetics of a betayadokarivirus YkV3 in *R. necatrix* (Fig. 1), which revealed some features unique to YkV3 and common to YkV1. Furthermore, our group is the first to infect *C. parasitica*, the representative model filamentous fungus for mycovirus studies (Eusebio-Cope et al., 2015), with a yadokarivirus and its capsid donor (Fig. 2). Yadokariviruses rely on their partner dsRNA viruses for replication; hence, for a yadokarivirus to replicate in a given host fungus, its dsRNA virus partner must be able to replicate in the same host. Our attempts to transfect *C. parasitica* with YkV1 and its capsid donor YnV1 have been unsuccessful (unpublished data), and thus YkV3/RnMBV3 can be a model to study the yadokari/yadonushi nature in *C. parasitica*.

By reverse genetics of YkV3 in *R. necatrix*, we attempted to address two questions regarding (1) the sequence heterogeneity at the 5′-terminus found in some betayadokariviruses; and (2) the C-terminal part of unknown function encoded by both alphayadokariviruses and betayadokariviruses. The mutational analysis of the first nucleotide in the YkV3 genome revealed that the 5′-terminus could be plastically interchangeable between C or U (Fig. 1). This contrasts the situation of other (+)RNA viruses of the phylum *Kitrinoviricota* that require a precise nucleotide residue at the 5′-terminus (Boyer and Haenni, 1994; Lindenbach, 2022). Generally, the presence of non-viral nucleotides at the 5′ end is detrimental to virus infectivity because it serves as part of the *cis*-acting element of RNA replication (Esteban and Fujimura, 2003). The heterogeneity at the terminal ends has been observed not only in YkV3 and other betayadokariviruses, but also in unrelated dsRNA viruses (quadriviruses, family *Quadriviridae*, order *Ghabrivirales*) (Arjona-Lopez et al., 2018; Chiba et al., 2018). The RdRPs of these fungal viruses likely tolerate the sequence variability in their RNA recognition during viral RNA replication. This notion would also be supported by the infectivity of the YkV4b cDNA clone without ribozymes (Sato et al., 2022a). In addition to the flexibility in the terminal nucleotide, we observed flexibility inside the 3′-UTR sequence of YkV3 (Fig. S3), reminiscent of the case of poliovirus 1 (Brown et al., 2005) that belongs to the same order as yadokariviruses (*Pisuviricota*). In contrast to such flexibility in the non-coding parts, analysis of mutant $YkV3_{2Astop}$ revealed the indispensability of the putative C-terminal portion of the RdRP polyprotein likely liberated by the 2A-like peptide (Fig. 1). We have previously shown with an array of diverse YkV1 mutants that RdRP must be released from the polyprotein via 2A-like self-cleavage activity and that the C-terminal portion must be expressed for YkV1 replication (Das et al., 2021). The combined results suggest that an alphayadokarivirus YkV1 and a betayadokarivirus YkV3 commonly require this protein despite the low sequence conservation (Fig. S2), while its role in replication remains elusive.

The replication competency of YkV3/RnMBV3 in *C. parasitica* allowed us to investigate whether YkV3/RnMBV3 are targeted by antiviral RNA silencing. The much greater accumulation of RnMBV3 in an RNA silencing–deficient mutant (Δ*dcl2*) than in an RNA silencing-competent wild-type strain (EP155) suggests that RnMBV3 is targeted by RNA silencing (Fig. 4). The same pattern was also observed for YkV3 in the presence of RnMBV3, indicating that YkV3 may be directly targeted by RNA silencing in wild-type EP155 (Fig. 4). Alternatively, the reduced YkV3 accumulation may be a secondary consequence of the possible reduction in RnMBV3 replication followed by reduced supply of its capsids to YkV3. Even in the Δ*dcl2* strain, the accumulation level of RnMBV3 and YkV3 was still lower than that in the original host *R. necatrix* (Fig. 2B), which has functional RNA silencing machinery (Yaegashi et al., 2016). This contrasts with other heterologous viruses, including Rosellinia necatrix megabirnavirus 1 (RnMBV1), transferred to *C. parasitica* Δ*dcl2* that show levels of accumulation comparable to that in their original host *R. necatrix* (Chiba et al., 2013; Salaipeth et al., 2014). Besides the difference in viral accumulation level between *R. necatrix* and *C. parasitica*, we observed differences in the symptom induction by RnMBV3 and YkV3 between these two hosts. Neither RnMBV3 nor RnMBV3 plus YkV3 changes *in vitro* growth of *R. necatrix* (Sato et al., 2022a), whereas RnMBV3 caused abnormal growth of *C. parasitica* Δ*dcl2*, regardless of the presence of YkV3 (Fig. 3). Symptom induction specifically occurring in the experimental host *C. parasitica* by several other dsRNA viruses of *R. necatrix* has been noted in prior studies (Chiba et al., 2013, 2016; Salaipeth et al., 2014). We have recently identified master transcription regulators governing symptom expression in *Neurospora crassa* and other major ascomycetes (S. Honda and N. Suzuki, submitted). It will be of interest to examine whether the master regulators are involved in the observed symptomatic and asymptomatic infections in *C. parasitica* and *R. necatrix*.

The analysis in *C. parasitica* revealed the possibility of host-specific antagonism between YkV3 and RnMBV3. In the original host fungus *R. necatrix*, YkV3 has no impact on its partner RnMBV3, unlike other yadokariviruses that either promote or interfere with the replication of their capsid donor dsRNA viruses (Sato et al., 2022a; Zhang et al., 2016). In the newly established host fungus *C. parasitica*, at least the RNA silencing–deficient Δ*dcl2* strain, co-infection by YkV3 resulted in reduced accumulation of RnMBV3 (Fig. 2C). This may suggest competition over the RnMBV3 capsids between the two viruses. Note that there is a large variation in the accumulation level of RnMBV3 among transfectants irrespective of the presence or absence of YkV3. The possibility of host-specific antagonism between YkV3 and RnMBV3 should be validated in a more controllable way, such as the YkV3 reverse genetics system like in Fig. 1 using the same RnMBV3 transfectants as a donor. To try to establish the YkV3 reverse genetics system in *C. parasitica*, we transformed the wild-type EP155 strain with the YkV3 infectious cDNA clone and co-cultured with the Δ*dcl2* strain transfected with RnMBV3 as a virus donor. With this strategy, however, we did not detect YkV3 replication and horizontal transmission to the RnMBV3 donor side, at least based on direct RT-PCR of mycelia (data not shown). This outcome is likely due to the inhibition of the replication of RnMBV3 and YkV3 by host RNA silencing in the YkV3 cDNA transformant side.

In conclusion, we have shown the usefulness of a genetically manipulable YkV3/RnMBV3 system to study the yadokari/yadonushi nature. One of the important unanswered questions concerning the yadokari/yadonushi nature is the molecular mechanism determining the partnership specificity between yadokariviruses and dsRNA viruses. The specificity could be determined by molecular interaction between the components of a yadokarivirus (RNA and/or RdRP) and a partner dsRNA virus (RNA and/or capsids comprised of capsid protein and RdRP) (Hisano et al., 2018; Sato et al., 2022a; Zhang et al., 2016). In this regard, RnMBV3 has the advantage of belonging to the relatively

well-characterized family *Megabirnaviridae* (Sato et al., 2019), compared with the other characterized capsid donors that belong to the proposed "Yadonushiviridae" and "Megatotiviridae" families or to the genus *Botybirnavirus* (family unassigned) (Sato et al., 2023). For example, the capsid structure of RnMBV1, a sister virus to RnMBV3, has been analyzed using high-resolution cryo-electron microscopy (Miyazaki et al., 2015; Wang et al., 2023). Although RnMBV1 does not support YkV3 replication (Sato et al., 2022a), this structural information of RnMBV1 can be useful to predict or analyze that of RnMBV3. The structural information combined with the reverse genetics of YkV3 can help to identify the mechanism of interaction between RnMBV3 capsids and YkV3 RNA/proteins. Such experimental systems will also be important to prove the hypothesis that yadokariviruses replicate inside the capsids of partner dsRNA viruses (Hisano et al., 2018; Kondo et al., 2022), and to disprove a counterhypothesis that yadokariviruses replicate in host membranous structures like other (+)RNA viruses. On the one hand, the genetic tractability and manipulability of *C. parasitica* might be advantageous for the identification of host factors involved in this virus–virus–host interaction. On the other hand, the considerably low level of both viruses in wild-type *C. parasitica* (Fig. 4) would impose a limitation for such studies.

Author contributions

Nobuhiro Suzuki, Conceptualization, Data curation, Investigation, Visualization, Methodology, Writing, Funding acquisition; Yukiyo Sato, Data curation, Investigation, Formal analysis, Visualization, Methodology, Writing, Funding acquisition; Sakae Hisano, Formal analysis, Investigation, Methodology. All authors discussed the results and commented on the manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare that they have no conflict.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2023.199155.

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