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Characterization of a new megabirnavirus that confers hypovirulence with the aid of a co-infecting partitivirus to the host fungus, *Rosellinia necatrix*

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ABSTRACT

A new virus termed Rosellinia necatrix megabirnavirus 2 (RnMBV2) was molecularly and biologically characterized. RnMBV2 was originally harbored in isolate W8 of R. necatrix co-infected with the previously reported virus Rosellinia necatrix partitivirus 1 (RnPV1). RnMBV2 has molecular features similar and different from precedent megabirnaviruses, Rosellinia necatrix megabirnavirus 1 (RnMBV1) and Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1). The two genomic segments of RnMBV2 (9.0-kbp dsRNA1 and 8.0-kbp dsRNA2) each possess two open reading frames (ORF1 and 2 on dsRNA1 and ORF3 and 4 on dsRNA2), with a well conserved 5′-long untranslated region (UTR) of 1.7–1.8 kb between the segments, and relatively short 3′-UTR. The RnMBV2 dsRNA1-coded capsid protein (CP) and RNA-dependent RNA polymerase (RdRp) show higher sequence identity to those of SsMBV1 than to those of RnMBV1, whereas the RnMBV2 ORF3-coded protein is more closely related to the counterpart of RnMBV1. No significant amino acid sequence similarity was detected among ORF4-coded sequences of the three megabirnaviruses. Virion transfection and co-culturing allowed for single and double infection of mycelial incompatible isolates W37 and W97 by RnMBV2 and/or RnPV1. Their comparative analyses showed RnMBV2 to be able to confer hypovirulence with the aid of a co-infecting RnPV1, while the individual viruses exhibited asymptomatic infections. Interestingly, RnPV1 accumulation appeared to be increased in co-infected fungal strain with two segments of RnMBV2 relative to singly infected fungal strains. Furthermore, the dispensability of RnMBV2 dsRNA2 was demonstrated to be similar to that of the other two megabirnaviruses.

1. Introduction

Mycoviruses are found in a broad range of fungal species in every group of true fungi (Ascomycetes, Basidiomycetes, and Deuteromycetes) and fungus-like organisms (Oomycetes) (Pearson et al., 2009). Approximately two thirds of mycoviruses have double-stranded RNA (dsRNA) genomes and are classified into six families, *Partitiviridae*, *Totiviridae*, *Chrysoviridae*, *Reoviridae*, *Quadriviridae*, and *Megabirnaviridae* (Ghabrial et al., 2015). Most mycoviruses show asymptomatic infections, but several viruses found in phytopathogenic fungi are known to reduce host virulence (Ghabrial et al., 2015; Pearson et al., 2009).

Multiple virus infection has been observed in many fungal species (Buck 1998); however, the interactions of multiple-infected viruses have not been fully elucidated. Multiple virus infection is associated with reduced virulence in several plant pathogenic fungi, e.g., *Ophiostoma novo-ulmi* (Buck and Brasier, 2002), *Rhizoctonia solani* (Tavantzis et al., 2002), and *Helminthosporium victoriae* (Ghabrial et al., 2002). In these studies, dsRNAs remained in mixture and the effect of each dsRNA was ambiguous. More rigorous methods for artificial infection are now available, including transformation using virus-expression vectors, transfection of in-vitro transcribed viral positive-sense (+), and single-stranded (ss) RNA. In addition, transfection of purified virus particles proved to be effective in studies using *Cryphonectria parasitica* (Ghabrial and Suzuki,

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2009). These techniques become powerful tools not only to clarify the effect of single virus infection, but also to study multiple infection. Double infection with Cryphonectria hypovirus 1 (CHV1) and a mycoreovirus (Mycoreovirus 1 (MyRV1)) was achieved in *C. parasitica* to elucidate their synergistic effects (Sun et al., 2006).

Rosellinia necatrix Prillieux causes white root rot disease on more than 400 plant species worldwide (Pliego et al., 2012). Mycoviruses in *R. necatrix* have been studied in detail, the goal being to control white root rot with the deleterious mycovirus (virocontrol) (Kondo et al., 2013). From approximately 1000 isolates of *R. necatrix* collected throughout Japan, it was found that 20% harbored dsRNAs varying in size and number of segments, with some of these showing potential as virocontrol agents (Arakawa et al., 2002; Ikeda et al., 2004). Diverse viral species have been found in *R. necatrix*, i.e., a mycoreovirus (Mycoreovirus 3, MyRV3) (Osaki et al., 2002; Wei et al., 2003, 2004), two partitiviruses (Rosellinia necatrix partitivirus 1 and 2 (RnPV1 and RnPV2)) (Chiba et al., 2013b; Sasaki et al., 2005), a megabirnavirus (Rosellinia necatrix megabirnavirus 1 (RnMBV1)) (Chiba et al., 2009), two quadriviruses (Rosellinia necatrix quadrivirus 1 and 2 (RnQV1 and RnQV2)) (Lin et al., 2012, 2013), a victorivirus (Rosellinia necatrix victorivirus 1 (RnVV1)) (Chiba et al., 2013a), and a new (+) ssRNA virus, Rosellinia necatrix fusarivirus 1 (RnFV1) (Zhang et al., 2014). Artificial infection of protoplasts with purified particles of most aforementioned viruses was achieved in *R. necatrix* (Chiba et al., 2009; Sasaki et al., 2006; Sasaki et al., 2007). With these techniques, combined with hyphal tipping, we revealed that single virus infection of MyRV3 and RnMBV1 caused hypovirulence in *R. necatrix* (Chiba et al., 2009; Kanematsu et al., 2004; Sasaki et al., 2007), whereas the partitiviruses, quadriviruses, victorivirus, and fusarivirus had no effects on the fungus (Chiba et al., 2013a; Lin et al., 2012; Sasaki et al., 2006; Zhang et al., 2014).

RnMBV1, a member of *Megabirnaviridae*, is one of the most promising hypovirulence factors, exhibiting strong capacity to reduce virulence (Chiba et al., 2009), stable infection, and higher transmission efficiency by hyphal anastomosis than another hypovirulence factor, MyRV3 (Yaegashi et al., 2011). RnMBV1 has two segments (dsRNAs 1 and 2), each with a long and short conserved untranslated region (UTR) in the 5′ and 3′ termini, respectively. dsRNA1 codes for capsid protein (CP) and putative RNA-dependent RNA polymerase (RdRp). The RdRp of RnMBV1 is expressed as fusion protein with CP by ribosomal -1 frameshift and incorporated into the capsid of 520 Å and composed of 60 asymmetric dimers in the $T = 1$ lattice (Chiba et al., 2009; Miyazaki et al., 2015; Salaipeth et al., 2014). dsRNA2 would also codes for two proteins, although their functions are unknown. The analyses of genome-rearranged RnMBV1 by Kanematsu et al. (2014) suggested that dsRNA2 enhanced viral accumulation and virulence. Recently, a new member of *Megabirnaviridae* was found in a hypovirulent strain of *Sclerotinia sclerotiorum* (Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1)) (Wang et al., 2015). SsMBV1 resembles RnMBV1 in having long and conserved UTR in the 5′ side and predicted ORFs in L1-dsRNA, but differs in the sequence and length of the conserved region of 3′-UTR. L1-dsRNA of SsMBV1 was infectious and persisted in the host without dsRNA2 or rearranged segments as was indicated in genome rearranged RnMBV1-RS1 (Kanematsu et al., 2014). SsMBV1 was found to have slight or no effect when virus-free host isolates were transfected with purified virus particles (Wang et al., 2015).

The effects of multiple viral infection have not been studied in *R. necatrix,* although the fungus has shown various dsRNAs (multiple virus infections) in many single isolates (Arakawa et al., 2002; Ikeda et al., 2004). *R. necatrix* isolate W8 exhibits an irregular colony margin, slow growth rate on potato dextrose agar (PDA), and moderate virulence (Sasaki et al., 2005). The isolate contains three dsRNAs that can be separated by electrophoresis, i.e., L1-, L2-, and M-dsRNAs. The M-dsRNA contains two RnPV1 segments of almost the same size, each encoding RdRp and CP. L-dsRNAs are regarded as a distinct viral species from RnPV1 by their size and sequence similarity. Owing to the difficulties in curing isolate W8 of viral infection and in the separation of virus species, it has not been clear whether the moderate virulence of isolate W8 was ascribed to its intrinsic nature, or to L-dsRNAs. Here, we report that L1 and L2-dsRNA, hereafter termed dsRNA1 and dsRNA2, are the genome segments of a new virus, Rosellinia necatrix megabirnavirus 2 (RnMBV2), belonging to the family *Megabirnaviridae* and that the virus is likely to enhance accumulation of RnPV1. Coexistence of RnMBV2 and RnPV1 caused reduced mycelial growth and virulence of the host fungus, *R. necatrix*.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Profiles of isolates and strains are summarized in Table 1. Three isolates of *R. necatrix* were used. Isolates W8, W37, and W97 were obtained from Okayama, Chiba, and Saga Prefectures in Japan, respectively, and belonged to different mycelial compatibility groups (MCGs; 5, 34, and 80 (Arakawa et al., 2002; Nakamura's unpublished data)). W8 contained four dsRNA segments (dsRNA1, dsRNA2 and RnPV1), and isolates W37 and W97 were virus-free. W37hyg and W97hyg were transformed strains with the bacterial hygromycin resistance gene as previously described (Sasaki et al., 2006). RnPV1 was identified as a partitivirus (Sasaki et al., 2005), and dsRNA1 and dsRNA2 were recovered as a second megabirnavirus, i.e., RnMBV2 in this study. W37/RnPV1, W37hyg/RnMBV2, W37/RnMBV2 + RnPV1, W97/RnPV1, and W97/RnMBV2 (dsRNA1) + RnPV1 were virus-infected strains. Mycoviruses transfected or transmitted were indicated after the slash. W37/RnPV1 and W97/RnPV1 were obtained by transfection of RnPV1 particles (Sasaki et al., 2006). W97/RnMBV2 (dsRNA1) + RnPV1 was derived from a strain during the RnPV1 transfection assay, and infecting RnMBV2 lacked dsRNA2. W37hyg/RnMBV2 was obtained by dual culture with W37hyg and W8 (in this paper). W37/RnMBV2 + RnPV1 was obtained by dual culture with W37/RnPV1 and W37hyg/RnMBV2 (see Supplemental Fig. S1). All isolates and strains were grown on PDA (DIFCO laboratories, Detroit, USA) at 24 °C with or without hygromycin and kept at 4 °C until use. Mycelia for dsRNA extraction were cultured on cellophane-overlaid PDA dishes.

2.2. Viral introduction by dual culture

Virus-infected mycelial plugs were inoculated in pairs with virus-free plugs that had been transformed with a bacterial hygromycin B-resistance gene. Co-cultures were incubated at 24 °C for 15 days on 12-cm square dishes containing 30 ml of PDA. W8 was slow-growing relative to W37 and W97, so W8 was inoculated 3 days earlier, similar to the previous MyRV3 transmission assay (Sasaki et al., 2007). Mycelial plugs were removed mostly from colony margins and subcultured on PDA with or without hygromycin B for 5 days after inoculation. Subcultures were subjected to dsRNA extraction.

2.3. Extraction of total and dsRNA

Mycelia were collected from PDA with cellophane (cellophane-mycelia) and stored at −20 °C. Cellophane-mycelia were disrupted with a Multi-beads-shocker (Yasui Kikai, Osaka, Japan). Disruption tubes (3 ml) containing cellophane-mycelia and a metal-weight were pre-chilled with liquid nitrogen and shaken at 2500 rpm for 15 s. The treatment was repeated once after cooling with liquid