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A mycoreovirus suppresses RNA silencing in the white root rot fungus, *Rosellinia necatrix*

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ABSTRACT

RNA silencing is a fundamental antiviral response in eukaryotic organisms. We investigated the counterdefense strategy of a fungal virus (mycovirus) against RNA silencing in the white root rot fungus, *Rosellinia necatrix*. We generated an *R. necatrix* strain that constitutively induced RNA silencing of the exogenous green fluorescent protein (GFP) gene, and infected it with each of four unrelated mycoviruses, including a partitivirus, a mycoreovirus, a megabirnavirus, and a quadrivirus. Infection with a mycoreovirus (*R. necatrix* mycoreovirus 3; RnMyRV3) suppressed RNA silencing of GFP, while the other mycoviruses did not. RnMyRV3 reduced accumulation of GFP-small interfering (si) RNAs and increased accumulation of GFP-double-stranded (ds) RNA; suggesting that the virus interferes with the dicing of dsRNA. Moreover, an agroinfiltration assay *in planta* revealed that the S10 gene of RnMyRV3 has RNA silencing suppressor activity. These data corroborate the counterdefense strategy of RnMyRV3 against host RNA silencing.

Introduction

RNA silencing is a homology-dependent RNA degradation mechanism that is widely conserved among eukaryotic organisms (Cogoni, 2001; Hannon, 2002; Zamore, 2002; Baulcombe, 2004). This mechanism is triggered by the dicing of double-stranded RNA (dsRNA) into 21- to 25-nucleotide (nt) small interfering RNAs (siRNA) by an RNaseIII-like enzyme called Dicer. These siRNAs are loaded into Argonaute (AGO) proteins, and the siRNA-AGO complex [called the RNA-induced silencing complex (RISC)] degrades target RNAs in a sequence-specific manner (Meister and Tuschl, 2004). In some organisms including nematodes, plants, and fungi, the effect of RNA silencing is amplified by a host RNA-dependent RNA polymerase (RdRp), which converts single-stranded (ss) RNA into dsRNA and thereby promotes accumulation of secondary siRNA (Baulcombe, 2007; Dang et al., 2011).

One of the important biological roles of RNA silencing is as a fundamental defense system against viral infections (Wang and Metzlaff, 2005; Ding and Voinnet, 2007). Accumulations of dsRNAs as viral genomes or replication intermediates and highly structured viral ssRNAs are thought to be capable of inducing RNA silencing, thus inhibiting virus propagation in host organisms. To establish a viral infection, many viruses have evolved a counterdefense strategy against RNA silencing. In general, viruses encode a protein that can suppress RNA silencing, called an RNA silencing suppressor (RSS). A number of viral RSSs have been identified among plant and animal viruses (Roth et al., 2004; Voinnet, 2005; Bivalkar-Mehla et al., 2011). These target various steps in RNA silencing, including siRNA generation, siRNA loading into the RISC, assembly of the RISC, and slicing of target RNA (Ding and Voinnet, 2007).

Most fungal viruses (referred to as mycoviruses), including viruses belonging to the Partitiviridae, the Totiviridae, the Chrysoviridae, and the Reoviridae, have dsRNA genomes; however, ssRNA mycoviruses, including those in the Hypoviridae, Narnaviridae, and Endornaviridae, also accumulate dsRNAs as replication intermediates (Pearson et al., 2009; Ghabrial and Suzuki, 2009). These mycoviral RNAs are thought to be potential targets of RNA silencing; nevertheless, limited studies have been reported of the counterstrategies of mycoviruses against host RNA silencing. It has been reported that *Cryphonectria* hypovirus 1 (CHV1) suppresses RNA silencing in the host fungus *Cryphonectria parasitica* by expression of an RSS, papain-like protease P29, encoded by the virus (Segers et al., 2006,2007). Another example of a mycovirus suppressing RNA silencing has been observed in *Aspergillus nidulans* (Hammond et al., 2008). Infection by *Aspergillus* virus 1816 suppressed RNA silencing in *A. nidulans*, but infection with *Aspergillus* virus 178 or *Aspergillus* virus 341 did not. Other research reported that a dsRNA mycovirus belonging to the Totiviridae, *Magnaporthe oryzae* virus 2 (MoV2), did not suppress

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RNA silencing in *M. oryzae*, and a significantly lower level of MoV2-derived siRNAs accumulated (Himeno et al., 2010). The authors suggested that a novel strategy allowed MoV2 to evade host RNA silencing.

Rosellinia necatrix is a soil-borne phytopathogenic filamentous fungus. The fungus causes white root rot in a wide range of herbaceous and woody plants. Because white root rot disease is lethal, it has considerable economic impact. This is especially true in the cultivation of fruit trees, including apples, Japanese pears, and grapes, in Japan. For over 10 years, our group has studied the mycoviruses of *R. necatrix* with the hope of developing a mycovirus-mediated biological control of white root rot (called a virocontrol; Chiba et al., 2009; Ghabrial and Suzuki, 2009). A variety of mycovirus-related dsRNAs have been found in the over 1000 *R. necatrix* isolates collected (Arakawa et al., 2002; Ikeda et al., 2005). At present, six mycoviruses have been identified in this collection, including *R. necatrix* partitivirus 1 (RnPV1), RnPV2, *R. necatrix* mycoreovirus 3 (RnMyRV3), *R. necatrix* megabirnavirus 1 (RnMBV1), *R. necatrix* quadrivirus 1 (RnQV1), and *R. necatrix* victorivirus 1 (RnVV1) (Sasaki et al., 2005; Wei et al., 2004; Chiba et al., 2009; Lin et al., 2012; Chiba et al., 2011, 2013a,2013b). These six mycoviruses, RnPV1, RnPV2, RnMyRV3, RnMBV1, RnQV1, and RnVV1, have non- or multi-segmented dsRNA genomes (one segment in RnVV1, two in RnPV1, RnPV2, and RnMBV1, four in RnQV1, and 12 in RnMyRV3), and they are classified in the families Partitiviridae, Reoviridae, Megabirnaviridae, Quadriviridae, and Totiviridae, respectively. RnPV1, RnPV2, RnQV1, and RnVV1 infections do not cause obvious phenotypic changes in *R. necatrix* (Sasaki et al., 2005; Lin et al., 2012; Chiba et al., 2011, 2013a,2013b). However, RnMyRV3 and RnMBV1 infections significantly reduce the mycelial growth and virulence of *R. necatrix* (Kanematsu et al., 2010; Chiba et al., 2009), indicating that the latter two mycoviruses are possible virocontrol agents. These *R. necatrix* mycoviruses are different in their genomic organization and biological impact on *R. necatrix*. It is of great interest to determine whether these different mycoviruses have evolved counterdefense strategies against RNA silencing in *R. necatrix*.

We generated a *R. necatrix* strain that constitutively induces RNA silencing of the exogenous green fluorescent protein (GFP) gene by expression of the dsRNA of GFP. The GFP-silencing strain was infected with each of the four mycoviruses (RnPV1, RnMyRV3, RnMBV1, and RnQV1) to examine whether the mycoviruses might have the ability to suppress RNA silencing. We found that RnMyRV3 infection suppressed dsRNA-induced silencing of GFP by interfering with the dicing of dsRNA into siRNA. In addition, an agroinfiltration assay in *Nicotiana benthamiana* revealed that the S10 gene of RnMyRV3 has RSS activity. These data give new light on the counterdefense strategies of mycoviruses against host RNA silencing.

Results

Induction of RNA silencing in R. necatrix

To induce RNA silencing of the GFP gene in *R. necatrix*, a GFP-expressing strain (RGFP) was generated by transformation of a W97 isolate with the plasmid pCPGFP, followed by the transformation of the RGFP strain with the plasmid pII99-dsGFP, which expresses the double-stranded (ds) form of GFP-RNA. The resulting strain was designated RiGFP. Colony growth and morphology of RGFP and RiGFP were comparable to those of the parental W97; however, no GFP fluorescence was found in the mycelia of RiGFP, in contrast to the strong GFP fluorescence of RGFP (Fig. 1A). Northern blot analysis detected an accumulation of GFP-mRNA in RGFP that was markedly reduced in RiGFP (Fig. 1B). Conversely,

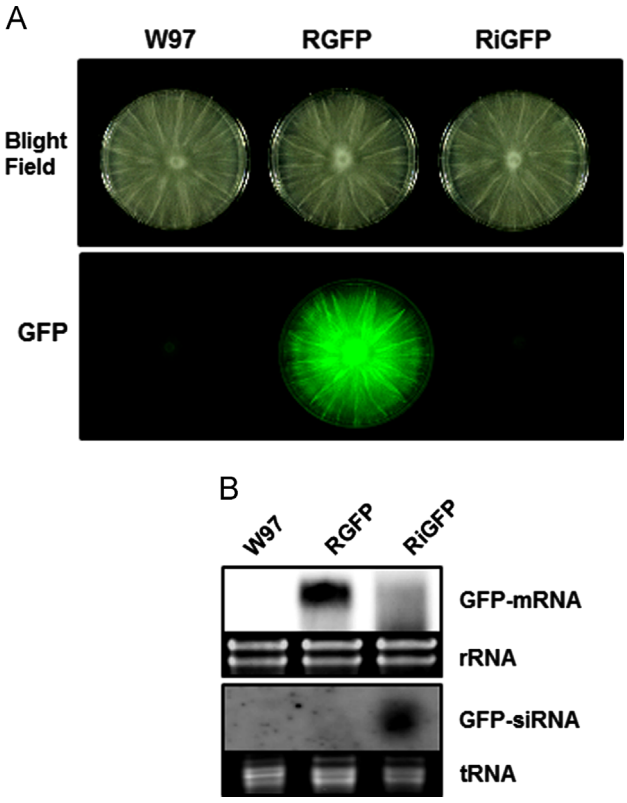

Fig. 1. Generation of *Rosellinia necatrix* GFP-silencing strain. (A) Bright-field (top) and GFP (bottom) images of the parental W97 isolate, GFP-expressing W97 strain (RGFP), and GFP-silencing W97 strain (RiGFP) colonies after 6 days of culturing. (B) Northern blot analysis of GFP-mRNA and siRNA in the RiGFP strain. Ethidium bromide stainings of rRNA and tRNA are shown as gel-loading controls, respectively.

a hallmark of the induction of RNA silencing, a 20–22 nt small interfering (si) RNA of GFP, was detected in RiGFP but not in RGFP (Fig. 1B). These results indicate that the disappearance of GFP fluorescence in RiGFP was due to the induction of RNA silencing of the GFP. This is the first report of induced RNA silencing of an exogenous gene in *R. necatrix*.

Suppression of RNA silencing in R. necatrix by mycoreovirus infection

To investigate whether four mycoviruses, a partitivirus (RnPV1-W8), a mycoreovirus (RnMyRV3-W370), a megabirnavirus (RnMBV1-W779), and a quadrivirus (RnQV1-W1075), might suppress RNA silencing in *R. necatrix*, RiGFP and W97 strains infected with each of the four mycoviruses (W97-par, -reo, -bir, -qua) were dual-cultured for virus transmission to the recipient RiGFP via hyphal fusion (anastomosis). In the dual-culturing of RiGFP with W97-par, W97-bir, or W97-qua, no GFP fluorescence was found in the recipient RiGFP colonies after 7 and 12 days in culture (data not shown). Infection of the RiGFP recipient by each mycovirus was confirmed by dsRNA analysis of sub-cultured mycelia (data not shown), indicating that these three mycoviruses, RnPV1-W8, RnMBV1-W779, and RnQV1-W1075, do not suppress silencing of GFP in the RiGFP strain. In the case of RnMyRV3-W370, GFP fluorescence was found in a small area of the RiGFP recipient at the interface between colonies after 7 days of culturing, and the area showing GFP fluorescence was enlarged within the RiGFP recipient after 12 days of culturing (Fig. 2A). Similar results were obtained in the case of other RnMyRV3 isolates (RnMyRV3-W713, -W720, -W780, -W966; data not shown). A RiGFP strain infected with RnMyRV3-W370 (RiGFP-reo) was derived by sub-culturing of mycelia showing GFP fluorescence in the RiGFP recipient. RiGFP-reo colony growth was slower than that of RGFP and

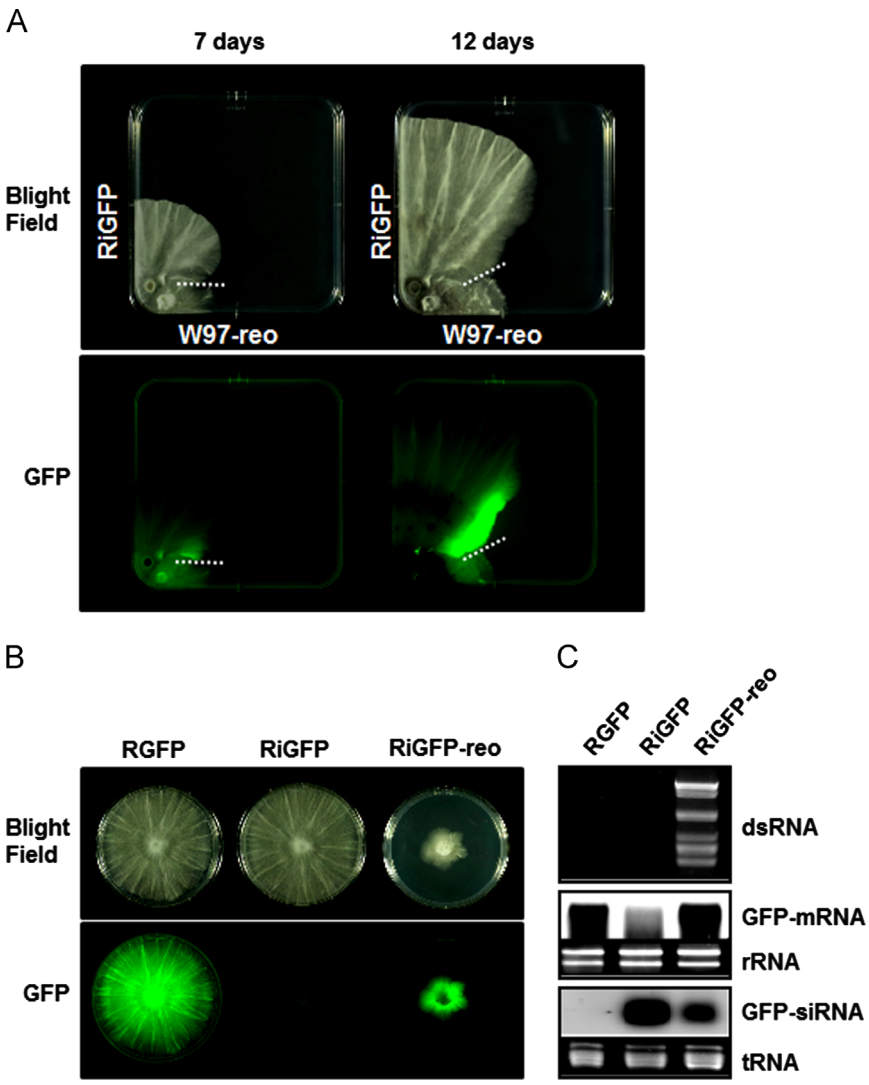

Fig. 2. Suppression of GFP silencing in *R. necatrix* by RnMyRV3 infection. (A) Bright-field (top) and GFP (bottom) images of the W97 strain infected with RnMyRV3 (W97-reo; donor) and GFP-silencing W97 strain (RiGFP; recipient) paired colonies after 7 and 12 days of culturing. Dashed lines indicate the interface between the colonies. (B) Bright-field (top) and GFP (bottom) images the GFP-expressing W97 strain (RGFP), RiGFP strain, and RiGFP strain infected with RnMyRV3 (RiGFP-reo) colonies after 6 days of culturing. (C) dsRNA analysis of RnMyRV3 and northern blot analysis of GFP-mRNA and -siRNA. RNA samples were extracted from 6-day-old cultured mycelia of RGFP, RiGFP, and RiGFP-reo. Ethidium bromide stainings of rRNA and tRNA are shown as gel-loading controls, respectively.

RiGFP (Fig. 2B), and infection by RnMyRV3 in RiGFP-reo was confirmed by dsRNA analysis (Fig. 2C). This is consistent with a previous report of growth reduction in *R. necatrix* strains infected with RnMyRV3 (Kanematsu et al., 2004). The GFP fluorescence found in the RiGFP-reo colony was as strong as that of RGFP (Fig. 2B). Northern blot analysis showed that the level of GFP-mRNA accumulated in RGFP and RiGFP-reo was higher than that in RiGFP (Fig. 2C). Conversely, the accumulation of GFP-siRNA in RiGFP-reo was reduced relative to the accumulation in RiGFP, but not completely eliminated (Fig. 2C). Collectively, these results demonstrated that RnMyRV3 infection suppresses RNA silencing in *R. necatrix*. This is the first report of suppression of RNA silencing by a mycoreovirus.

RnMyRV3 interferes with siRNA generation

It is known that a host RNA-dependent RNA polymerase (RdRp) converts ssRNA into dsRNA, and thereby generates secondary siRNA in fungi (Dang et al., 2011). Because RiGFP expresses both ssRNA and dsRNA of GFP, both primary and secondary GFP-siRNA must be accumulated. Therefore, it is unclear whether the reduced accumulation of GFP-siRNA in RiGFP-reo (shown in Fig. 2C) is due to interference with the dicing of dsRNA into siRNA, or with the pathway of secondary siRNA generation. To address this question, W97 strain was transformed with the plasmid pII99-dsGFP. The resulting strain, designated irGFP, expresses GFP-dsRNA that is processed into GFP-siRNA. The irGFP strain was dual-cultured with W97-reo to generate the RnMyRV3-infected irGFP strain (irGFP-reo). Northern blot analysis showed that the accumulation level of GFP-siRNA in 5 μg of the sample from irGFP-reo was lower than that in 1 μg of the sample from the virus-free irGFP strain (Fig. 3A), indicating that RnMyRV3 infection reduced GFP-siRNA accumulation below 5-fold in the irGFP strain. To find out if RnMyRV3 increases accumulation of GFP-dsRNA, we carried out northern blot analysis of GFP-dsRNA. The dsRNA samples were obtained by digestion of high-molecular weight RNA with S1 nuclease, and, by agarose gel electrophoresis and ethidium bromide staining, we confirmed elimination of ssRNA in all samples and presence of RnMyRV3-dsRNA in irGFP-reo (Fig. 3B, top panel). Northern blot analysis of the dsRNA samples showed that accumulation level of GFP-dsRNA in irGFP-reo was higher than that in virus-free irGFP

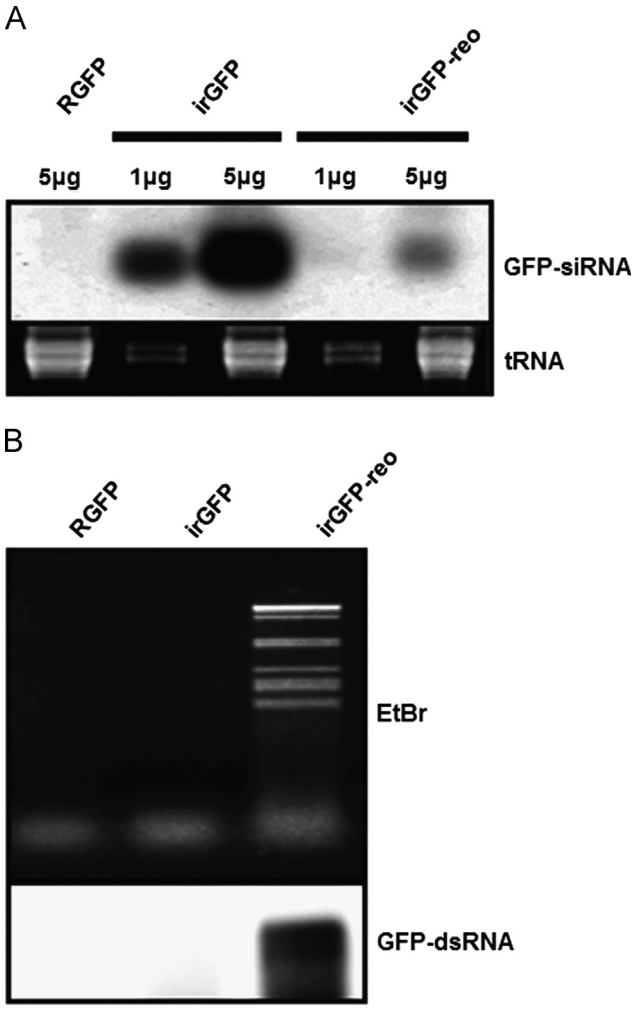

Fig. 3. The effect of RnMyRV3 infection on siRNA accumulation. (A) Northern blot analysis of GFP-siRNA from GFP-expressing (RGFP) and GFP-dsRNA-expressing W97 strain (irGFP), and irGFP strain infected with RnMyRV3 (iRGFP-reo). Ethidium bromide staining of tRNA is shown as gel-loading controls. (B) Northern blot analysis of GFP-dsRNA in RGFP, irGFP, and irGFP-reo. 20 μg of HMW-RNA samples were digested with S1 nuclease, absence of ssRNA and presence of dsRNA of RnMyRV3 are confirmed by ethidium bromide staining of agarose gels.

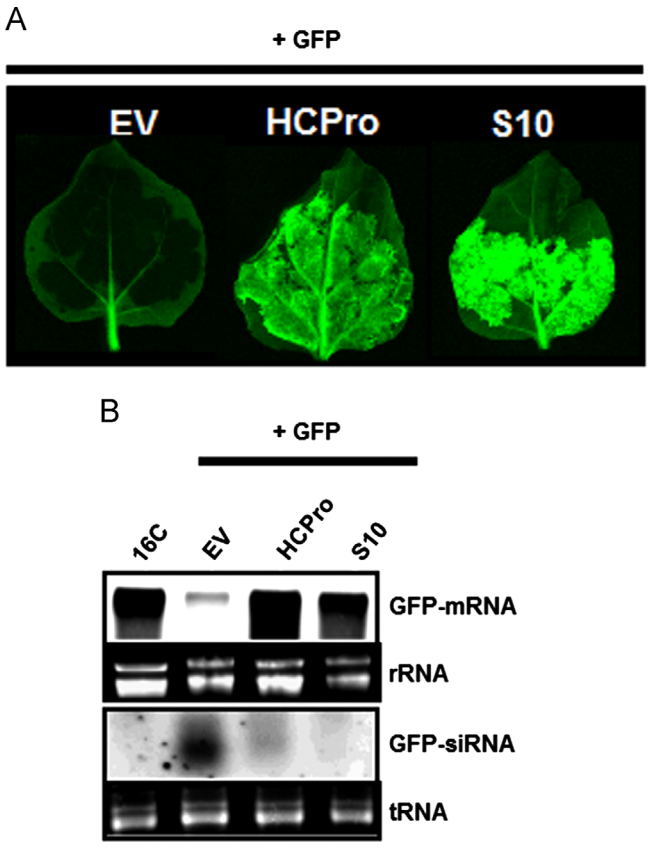

Fig. 4. RNA silencing suppression of the S10 gene of RnMyRV3 in *Nicotiana benthamiana*. (A) GFP fluorescence in leaves of the *N. benthamiana* line 16c agroinfiltrated with a mixture of agrobacteria carrying pBE2113-GFP (GFP); the construct is indicated at the top of each image at 5 days post-infiltration (dpif). (B) Northern blot analysis of GFP-mRNA and -siRNAs extracted from the infiltrated regions of leaves of *N. benthamiana* line 16c at five dpif. Lane 16C indicates samples from non-agroinfiltrated leaves *of N. benthamiana* line 16 c. Ethidium bromide staining of rRNA and tRNA are shown as gel-loading controls, respectively.

(Fig. 3B; bottom panel). Collectively, these results strongly suggest that RnMyRV3 interferes with the dicing of dsRNA into siRNA.

S10 gene of RnMyRV3 suppresses RNA silencing in planta

Many animal and plant viruses encode RSSs to counteract against host RNA silencing (Roth et al., 2004; Voinnet, 2005; Bivalkar-Mehla et al., 2011). Identification of RSS genes among several candidate virus genes has been commonly conducted using an agroinfiltration assay in *N. benthamiana* line 16C (Brigneti et al., 1998). We used an agroinfiltration assay to test whether 12 genes (S1–S12; putative protein coding region) in each respective genome segment of RnMyRV3-W370 might have RSS ability. An agrobacterial strain carrying each construct was abbreviated as expressing a gene, such as GFP, empty vector (EV), HCPro, or S1–S12. GFP fluorescence was no longer visible at 5 days post-infiltration (dpif) in *N. benthamiana* leaves agroinfiltrated with GFP plus EV (Fig. 4A). In contrast, strong GFP fluorescence was found in leaves agroinfiltrated with GFP plus HCPro (Fig. 4A). Among the S1 to S12 genes, strong GFP fluorescence was found only in leaves agroinfiltrated with GFP plus S10 at five dpif, similar to HCPro (Fig. 4A). In contrast, GFP fluorescence was no longer visible in leaves agroinfiltrated with GFP plus S1–S9, S11, or S12, similar to the empty vector (data not shown). Northern blot analysis showed that accumulation levels of GFP-mRNA in leaves agroinfiltrated with GFP plus HCPro or S10 were higher than the accumulation with GFP plus EV (Fig. 4B). Conversely, the accumulation of GFP-siRNA in leaves agroinfiltrated with GFP plus HCPro or S10 was markedly reduced relative to that in leaves with GFP plus EV (Fig. 4B). These results indicate that the S10 gene of RnMyRV3 functions as a RSS in *N. benthamiana*.

Detection of S10 gene products in R. necatrix infected with RnMyRV3

The S10 gene (nt 166–1098) is predicted to encode an approximately 34 kDa protein (310 aa). We performed immunoblot analysis of the *R. necatrix* W97 strain infected with RnMyRV3-W370 (W97-reo) using two antibodies against independent polypeptide sequences in the 310 aa of the S10 gene (S10PN: 51–69 aa; S10PC: 287–306 aa). The results revealed that the S10PN antibody specifically detected two proteins of approximately 32 and 20 KDa (P32 and P20), while the S10PC antibody specifically detected P32, an 11 kDa protein (P11), and some faint bands at slower migrations (Fig. 5). Because P32 was detected by both antibodies and was nearly equal to the predicted molecular weight of the S10 gene (34 kDa), it was concluded that P32 is the full-length S10 gene product. P20 and P11 are C-terminal and N-terminal truncated products, respectively. On the other hand, both antibodies detected the single band in *N. benthamiana* leaves transiently expressing the S10 gene by agroinfiltration at modestly faster

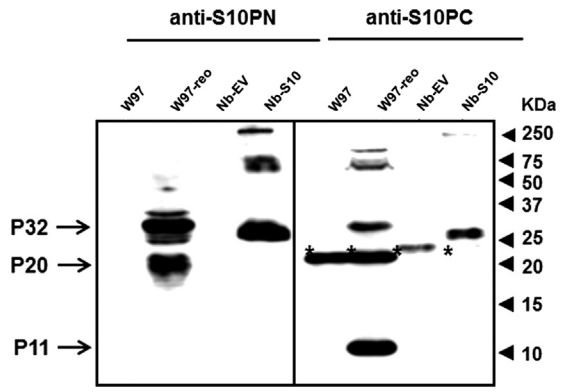

Fig. 5. Immunoblot analysis of S10 gene products from RnMyRV3. Total proteins were extracted from 6-day-old cultured mycelia of W97 and W97 infected with RnMyRV3(W97-reo), and from *N. benthamiana* line 16c leaves agroinfiltrated with empty vector (Nb-EV) or vector expressing the S10 gene (Nb-S10). Two antibodies, anti-S10PN and anti-S10PC, were raised against 51–69 aa and 287–306 aa, respectively, of the 310 aa S10 gene sequence. Arrows indicate the positions of the S10 gene products including P32, P20, and P11. Arrowheads indicate the positions of protein molecular markers. Asterisks indicate non-specific bands.

migration than P32; indicating that P20 and P11 are specific products in *R. necatrix* infected with RnMyRV3.

Discussion

RNA silencing is regarded as a fundamental defense system against virus infection in filamentous fungi (Segers et al., 2007), but the counterdefense strategy of mycoviruses against RNA silencing is largely unknown. We generated the *R. necatrix* strain RiGFP, in which RNA silencing of GFP is constitutively induced by expression of the ds-form of GFP-RNA (Fig. 1). We then investigated whether infection of the RiGFP strain with each of four unrelated mycoviruses [a partitivirus (RnPV1), a mycoreovirus (RnMyRV3), a megabirnavirus (RnMBV1), and a quadrivirus (RnQV1)] might suppress RNA silencing of GFP. Among these mycoviruses, we found that GFP fluorescence was reversed in the RiGFP strain infected with RnMyRV3 (RiGFP-reo) (Figs. 2A and B). The increased accumulation of GFP-mRNA and reduced accumulation of GFP-siRNA in RiGFP-reo demonstrated RNA silencing suppression by RnMyRV3 infection (Fig. 2C). This is the first report of RNA silencing suppression by a mycoreovirus. We have shown that a GFP-reversal assay is a convenient, non-destructive method of testing the silencing suppressor activity of candidate mycoviruses in *R. necatrix*.

We have previously shown that RnMyRV3 distributes unevenly in single fungal colonies (Yaegashi et al., 2011). Consistent with this finding, GFP fluorescence occasionally distributes unevenly in some colonies of RiGFP-reo. Sub-cultured mycelia from the regions of colonies lacking GFP expression were not infected with RnMyRV3 and therefore never showed GFP fluorescence (data not shown). These results indicate that suppression of RNA silencing is concomitant with RnMyRV3 infection. It has been reported that RnMyRV3 infection reduces the mycelial growth and virulence of *R. necatrix* (Kanematsu et al., 2004). Moreover, the virus can infect other fungi, including *C. parasitica*, *Valsa ceratosperma*, and *Diaporthe* sp., by protoplast-mediated artificial inoculation, and reduces the mycelial growth and virulence of these fungi (Kanematsu et al., 2010). In plant viruses, the ability to suppress RNA silencing is closely associated with virus pathogenicity (Wang et al., 2012). It will be of interest to investigate the association between suppression of RNA silencing and phenotypic alterations of host fungi by RnMyRV3 infection.

In contrast to RnMyRV3, three other mycoviruses, RnPV1, RnMBV1, and RnQV1, did not suppress RNA silencing in our experimental system. Hammond et al. (2008) reported that neither *Aspergillus* virus 178 nor *Aspergillus* virus 341 suppressed RNA silencing. Himeno et al. (2010) also reported that a dsRNA mycovirus belonging to the Totiviridae, *M. oryzae* virus 2 (MoV2), did not suppress RNA silencing in *M. oryzae* and significantly lowered the accumulation of MoV2-derived siRNA. Since many dsRNA viruses retain their genomes and RNA synthesis enzymes, and they replicate within their virions (Jacobs and Langland, 1996; Mertens, 2004), it is easy to envision that their encapsidated dsRNA genomes are sequestered from host RNA silencing machinery. Alternatively, dsRNA mycoviruses may have evolved other unknown strategies to escape from RNA silencing.

RnMyRV3 reduced the accumulation level of GFP-siRNA in both *R. necatrix* strains; the RiGFP strain that produces both primary and secondary GFP-siRNAs (Fig. 2C), and the irGFP strain that produces only primary GFP-siRNA (Fig. 3A). In addition, RnMyRV3 increased accumulation of GFP-dsRNA in the irGFP strain (Fig. 3B). These results strongly suggest that RnMyRV3 interferes with the dicing of dsRNA into siRNA. Because RnMyRV3 reduces but does not completely eliminate accumulation of GFP-siRNA in the two strains, the interference with the siRNA generation by RnMyRV3 would be leaky. This idea is consistent with our preliminary data using deep sequencing analysis, which shows that siRNAs are derived from 12 genome segments of RnMyRV3 (Yaegashi et al., unpublished data). On the other hand, recent studies have reported that a partitivirus (RnPV2) and a victorivirus (RnVV1) more effectively replicated in an RNA silencing-defective, dicer-like gene *dcl*2 knock-out mutant strain of *C. parasitica* (⊿dcl-2) than in the wild type strain of the fungus (Chiba et al., 2013a,2013b). These findings combined with our preliminary data emphasize that encapsidated dsRNA mycoviruses are also potential targets of host dicer-like proteins. It is noteworthy that a dsRNA virus belonging to the Reoviridae, such as RnMyRV3, has evolved the ability to interfere with the dicing of dsRNA. RnMyRV3 may be a preferential target of host dicer-like proteins. In *C. parasitica*, a dicer-like gene *dcl*2 and an argonaute-like gene *agl*2, are up-regulated in response to both mycovirus infection and expression of dsRNA (Zhang et al., 2008; Sun et al., 2009). Importantly, these papers have also reported that an RNA silencing suppressor, P29 of CHV1, represses the up-regulation of *dcl*2 and *agl*2. It is possible that RnMyRV3 infection may also repress the upregulation of dicer-like gene(s) in *R. necatrix* similar to CHV1 in *C. parasitica*. Alternatively, RnMyRV3 may impair the function of dicer-like protein(s) and/or sequester dsRNA, as reported for the B2 protein from Wuhan nodavirus (Qi et al., 2012). We have recently identified homologs of dicer-like, argonaute-like, and RdRp-like genes from the draft genome sequence of the *R. necatrix* W97 isolate (Shimizu and Kanematsu, unpublished data). Investigations into the potential roles of the RNA silencing-related genes against RnMyRV3 infection and the molecular mechanism of RNA silencing suppression by RnMyRV3 will provide further insights into the interactions between mycoviruses and host RNA silencing.

Many plant and animal viruses encode RSS genes to counteract against RNA silencing (Roth et al., 2004; Voinnet, 2005; Bivalkar-Mehla et al., 2011). Likewise, the S10 gene of RnMyRV3 showed RSS activity in *N. benthamiana* (Fig. 4). This is the second example of a RSS identified among mycoviruses, the first being P29 of CHV1 (Segers et al., 2006). Among the Reoviridae, Pns10 of the Rice dwarf virus (RDV; *Phytoreovirus* sp.) has also been identified to have RSS activity (Cao et al., 2005; Ren et al., 2010). There is no nucleotide or amino acid homology between the S10 gene of RnMyRV3 and the Pns10 gene of RDV, suggesting that these viruses independently acquired an RSS during their evolution. Phylogenic analysis supports the distant relationship between

RnMyRV3 and RDV (Wei et al., 2004). Importantly, it is unclear which segment of another mycoreoviral species, *C. parasitica* mycoreovirus 1 (MyRV1) is the counterpart of the S10 gene of RnMyRV3 (Suzuki et al., 2004). Among the 11 segments of MyRV1, no counterparts of segments S10 and S11 have been found from among the 12 segments of RnMyRV3 (Suzuki et al., 2004), raising the possibility that either of the two segments may encode a gene that is functionally homologous to the S10 gene of RnMyRV3. However, none of the 11 genes in the respective segments of MyRV1 showed RSS activity by an agroinfiltration assay in *N. benthamiana* (Dr. Nobuhiro Suzuki; personal communication). Therefore, it is thought that MyRV1 has no counterpart of the S10 gene of RnMyRV3, suggesting that RSS is not commonly conserved among *Mycoreovirus* species.

Unfortunately, no transformant was regenerated by protoplast-mediated transformation of the RiGFP strain, with either the S10 gene expression vector based on pCPXHY1, or the highly efficient auto-replication vector pAMA-H (Shimizu et al., 2012). We could not therefore demonstrate RSS activity of the S10 gene in *R. necatrix*. Transfection of the *R. necatrix* protoplasts with the RnMyRV3 virion was successful (Kanematsu et al., 2004), suggesting that constitutive expression of the S10 gene from a housekeeping gene promoter may affect the regeneration of the protoplast. Further study is required to determine whether transformation with the S10 gene expression vector is possible fungal species other than *R. necatrix*. However, accumulation of the S10 gene product (P32) in *R. necatrix* infected with RnMyRV3, and in *N. benthamiana* leaves transiently expressing the S10 gene, was demonstrated by immunoblot analysis (Fig. 5). Unexpectedly, in *R. necatrix* infected with RnMyRV3, N-terminal 20 KDa protein (P20) and C-terminal 11 KDa protein (P11) of P32 were also detected. This suggests that P32 is cleaved into P20 and P11, since the sum of molecular weights of P20 and P11 is nearly equal to P32. Post-translational protein cleavage has been reported for structural and non-structural proteins of avian reoviruses, including μB, σA, and μNS (Ji et al., 2010; Busch et al., 2011); however, the actual cleavage mechanisms of these proteins are unclear. Notably, P29 of CHV1 is generated by the autocatalytic cleavage of the precursor protein P69 (Choi et al., 1991). Future study will address the question as to whether P32 is also cleaved by autocatalysis or another mechanism, and which of the S10 protein cleavage products has the RSS activity in *R. necatrix*.

Materials and methods

Fungal strains and culture conditions

R. necatrix virus-free isolate W97 was used as the parental strain for fungal transformation. W97 strains infected with *R. necatrix* partitivirus 1 (RnPV1)-W8 (W97-par; Sasaki et al., 2006), *R. necatrix* mycoreovirus 3 (RnMyRV3)-W370 (W97-reo; Sasaki et al., 2007), *R. necatrix* megabirnavirus 1 (RnMBV1)-W779 (W97-bir; Chiba et al., 2009), or *R. necatrix* quadrivirus 1 (RnQV1)-W1075 (W97-qua; Lin et al., 2012) were used independently as the donor strains in virus transmission assays. All derivative strains were cultured on potato dextrose agar (PDA; Difco) plates with or without geneticin (150 mg/L) at 25 °C in the dark. Stocks of each strain were stored in 10% glycerol at −80 °C. For nucleic acid (DNA or RNA) and protein extractions from mycelia, strains were cultured on PDA (with or without geneticin) with sterilized cellophane seated on the surface for an appropriate time period. The cellophane containing the mycelia was stored at −80 °C prior to use. For virus transmission, donor and recipient strains were dual-cultured as described previously (Yaegashi et al., 2011). After 12 days, a mycelial agar piece from the recipient was sub-cultured on PDA containing the appropriate antibiotics, and dsRNA was extracted as described below, to confirm the viral infection.

Plasmids

For fungal transformation

For construction of a green fluorescent protein (GFP) expression vector without the hygromycin resistance gene, the plasmid pCPXHY1eGFP (Pliego et al., 2009) was digested with *Sal*I and *Bam*HI. This excised the hygromycin resistance gene, and the plasmid was self-ligated after a blunt-end treatment using T4DNA polymerase (Takara). The resulting plasmid was designated pCPGFP. A plasmid expressing the dsRNA of the GFP gene in hairpin form was constructed. First, for the spacer region, a 400 bp DNA fragment of the GUS gene (nt 600–1000) was amplified from pBI121 (Clontech) with two primers [gus600stu(+) and gus1000sph(−); Supplemental Table 1] by polymerase chain reaction (PCR) using an Expand Long Range Polymerase (Roche). This fragment was ligated downstream of the GFP sequence in pCPXHY1eGFP using *Stu*I and *Sph*I restriction sites. Subsequently, the full-length 720 bp DNA fragment of the GFP gene was amplified by PCR using two primers [GFPsph(+) and GFPsph(−); Supplemental Table 1]. This fragment was ligated in an inverted direction downstream of the spacer region in an intermediate plasmid using the *Sph*I restriction site. The resulting plasmid was designated pCPHdsGFP. The plasmid pII99 (harboring a geneticin-resistance gene expression cassette; Namiki et al., 2001) was digested with *Eco*RI, blunt-ended using T4 DNA polymerase (Takara), and dephosphorylated with bacterial alkaline phosphatase (BAP; Takara). The fragment containing the dsGFP expression cassette (nt 1656–5357) was obtained from pCPHdsGFP by digestion with *Sma*I, and the fragment was then ligated into the blunt-ended pII99 vector. The resulting pII99-based plasmid harboring the dsGFP expression cassette was designated pII99-dsGFP.

For agroinfiltration assay

Each putative protein coding region of the 12 segments in RnMyRV3-W370 (S1: 29–4111 nt; S2: 63–3743 nt; S3: 463–3270 nt; S4: 29–2206 nt; S5: 46–1986 nt; S6: 105–2000 nt; S7: 20–1468 nt; S8: 113–1090 nt; S9: 46–1188 nt; S10: 166–1098 nt; S11: 82–930 nt; and S12: 86–883 nt) was amplified by reverse-transcriptional(RT)-PCR from dsRNA of RnMyRV3-W370 using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and Expand Long Range PCR System (Roche) with specific primer pairs (Supplemental Table 1; both sense and antisense primers contained the appropriate restriction enzyme sites). Amplified cDNA fragments were first sub-cloned into the pGEM-T easy vector (Promega), and the resulting plasmids were designated pGtiS1, pGtiS2, pGtiS3, pGtiS4, pGtiS5, pGtiS6, pGtiS7, pGtiS8, pGtiS9, pGPtiS10, pGtiS11, and pGtiS12. The cDNA fragments from the sub-clones were ligated between the cauliflower mosaic virus 35 S promoter and the nopaline synthase terminator of the pBE2113 Ti plasmid (Mitsuhara et al., 1996) using appropriate restriction enzyme sites. The resulting Ti plasmids were designated pBE2113-S1, -S2, -S3, -S4, -S5, -S6, -S7, -S8, -S9, -S10, -S11, and -S12, respectively. For expression and induction of the RNA silencing of GFP, cDNA of GFP from pBI-GFP (Yaegashi et al., 2007) was cloned into the pBE2113 vector using *Xba*I and *Sac*I sites, and the resulting plasmid was designated pBE2113-mGFP. The empty vector (pBE2113-P35T) and the vector expressing HCPro of the clover yellow vein virus (pBE2113-HCPro) as previously reported (Yaegashi et al., 2007), were used as negative and positive controls, respectively. All Ti plasmids were introduced into *Agrobacterium tumefaciens* strain C58C1 by the freeze-thaw method.

Fungal transformation

To generate a GFP-expressing *R. necatrix* strain, the W97 isolate was transformed with pCPGFP by the protoplast-mediated polyethylene glycol (PEG)/$CaCl_2$ method, as described previously (Pliego et al., 2009). Mycelia expressing GFP fluorescence were identified under a fluorescence microscope, and the resulting GFP-expressing strain was designated RGFP. To induce silencing of GFP, RGFP was transformed again with pII99-dsGFP, and the resulting strain, resistant to geneticin and lacking GFP fluorescence, was designated RiGFP. W97 was transformed with pII99-dsGFP to generate a strain that expresses only dsRNA of GFP, designated irGFP. Fungal colonies were photographed under bright-field illumination using a digital camera (EOS1000D; Canon), and a GFP image of the colony was obtained using a LAS4000 image analyzer (GE Healthcare).

Agroinfiltration assay

The agroinfiltration assay in *N. benthamiana* line 16c (Brigneti et al., 1998) was done as described previously (Yaegashi et al., 2007). For co-infiltration, agrobacterial suspensions were prepared at $OD_{600}=1.0$, and equal volumes of each suspension were mixed prior to infiltration. Agroinfiltrated plants were kept in a growth chamber at 25 °C, in 16-h light/8-h dark conditions. GFP imaging of infiltrated leaves was done at 5 days after infiltration using a LAS4000 (GE Healthcare).

Northern blot analysis

Mycelial samples were homogenized using a Multi-Beads Shocker (Yasui Kikai), and high and low molecular weight (HMW or LMW) RNA were extracted using the mirVana miRNA Isolation kit (Ambion) according to the manufacturer's instructions. HMW-RNA and LMW-RNA samples were electrophoresed in 1% agarose gels containing 6% formaldehyde and 15% polyacrylamide gels containing 7 M urea, respectively, and the RNA was transferred to Hybond N+ nylon membrane (GE Healthcare) by capillary blotting using 20X SSC (3 M NaCl, 0.3 M sodium citrate). The membrane was fixed by UV-crosslinking and hybridized with DIG-labeled RNA probes (described below). The hybridized membrane was immunodetected with anti-DIG Fab fragments coupled with alkaline phosphatase (Roche). Chemiluminescence signals were developed using CDP-Star (GE Healthcare) and digitally visualized using an LAS4000 image analyzer (GE Healthcare).

For detection of GFP-RNA and GFP-small interfering (si) RNA in *R. necatrix* or *N. benthamiana*, we used a DIG-labeled antisense RNA probe of the EGFP sequence (720 nt; accession no. DQ768212) and the mGFP5 sequence (717 nt; accession no. U87973), respectively.

Immunoblot analysis

Polyclonal antibodies against a mixture of two independent polypeptides in the putative protein coding region of the S10 segment of RnMyRV3-W370 (S10P:nt 166–1098; 310 aa) were produced in rabbits by the Custom Antibody Production Service (Sigma-Aldrich, Japan). The two polypeptides used, S10PN (LPVSVPLNSQDVRPESGSD) and S10PC (VSEGFRSAYWSYVYASHGVC), correspond to 51–69 aa and 287–306 aa, respectively, of the 310 aa sequence of S10P. A mixture of these antibodies was purified using each S10PN or S10PC polypeptide-conjugated column to obtain each S10PN and S10PC polypeptide-specific antibody. Protein extraction and immunoblot analysis were carried out as described previously (Shimizu et al., 2012).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.07.010.

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