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

Hajime Yaegashi a,*, Nobuyuki Yoshikawab, Tsutae Itoa, Satoko Kanematsua

a Apple Research Station, National Institute of Fruit Tree Science, National Agriculture and Food Research Organization (NARO), 52 Shimokuriyagawa, Morioka, Iwate 020-0123, Japan
b Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

A R T I C L E   I N F O

Available online 27 July 2013

Keywords:
Mycovirus
Reovirus
dsRNA virus
RNA silencing
Viral suppressor
Rosellinia necatrix

A B S T R A C T

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 mycovirus (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.

© 2013 Elsevier Inc. All rights reserved.

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 Metzlafl, 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; Bivalk-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...
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 fungus. The fungus causes white root rot in a wide range of host RNA silencing.

Authors suggested that a novel strategy allowed MoV2 to evade counterdefense strategies against RNA silencing in *R. necatrix* with the hope of developing a mycovirus-mediated biological control of white root rot (called a virocontrol; Chiba et al., 2009; Chabrier 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 pl199-dsGFP, which expresses the double-stranded (ds) form of GFP-RNA. The resulting strain was designated RGFP. 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 RGFP, 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, 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. 1C). 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*, RGFP and W97 strains infected with each of the four mycoviruses (*W97-par*, *RnPv1*, *RnMyRV3*, *RnMBV1*, and *RnQV1*) were dual-cultured for virus transmission to the recipient RiGFP via hyphal fusion (anastomosis). In the dual-culturing of RiGFP with *W97-par*, *W97-par*, or *W97-qua*, no GFP fluorescence was found in the mycelia of RiGFP due to RNA silencing. In the case of *RnMyRV3–W370*, 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 when 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
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 plI99-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.
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 only in leaves agroinfiltrated with GFP plus HCPro (Fig. 4A). Among the S1 to S12 genes, strong GFP fluorescence was found 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...
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 RifGP, 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 RifGP strain with each of four unrelated mycoviruses [a paravirivirus (RnPV1), a mycoervirus (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 RifGP strain infected with RnMyRV3 (RifGP-reo) (Figs. 2A and B). The increased accumulation of GFP-mRNA and reduced accumulation of GFP-siRNA in RifGP-reo demonstrated RNA silencing suppression by RnMyRV3 infection (Fig. 2C). This is the first report of RNA silencing suppression by a mycovirus. 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 RifGP-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 ceratasperma, 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.

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 S10PN and S10PC, respectively, of the 310 aa S10 gene sequence. Arrowheads 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.

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 (Mv2), did not suppress RNA silencing in M. oryzae and significantly lowered the accumulation of Mv2-derived siRNA. Since many dsRNA viruses retain their genomes and RNA synthesis enzymes, and they replicate within their viroms (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 RifGP strain that produces both primary and secondary GFP-siRNAs (Fig. 2C), and the RifGP strain that produces only primary GFP-siRNA (Fig. 3A). In addition, RnMyRV3 increased accumulation of GFP-dsRNA in the RifGP 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 (RnP2V) and a victorious virus (RnV1V) more effectively replicated in an RNA silencing-defective, dicer-like gene dcl2 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 dcl2 and an argonaute-like gene agl2, 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 agl2. 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; Phytoereovirus 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. Phylogenetic 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. Nobuhito 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 for 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, sA, and μNS (Ji et al., 2010; Busch et al., 2011); however, the actual cleavage mechanisms of these proteins are unclear. Notably, P20 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 (RnP1)-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 quadrviruses 1 (RnQV1)-W1075 (W97-quar; Lin et al., 2012) were used independently as the donor strains for fungal transformation. All derivative strains were cultured on potato dextrose agar (PDA; Difco) plates with or without geneticin (150 mg/L) at 25 °C. For virus transmission, donor and recipient strains were dual-cultured as described previously (Yaegashi et al., 2011). After 12 days, a mycellial 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 (Piego et al., 2009) was digested with SalI and BamHI. 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 pBl121 (Clontech) with two primers [gus600stu(+)] and gus1000sp(+); 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 Stul and Spil restriction sites. Subsequently, the full-length 720 bp DNA fragment of the GFP gene was amplified by PCR using two primers [GFPsp(+)] and GFPsp(+); Supplemental Table 1]. This fragment was ligated in an inverted direction downstream of the spacer region in an intermediate plasmid using the Spil restriction site. The resulting plasmid was designated pcPHdsGFP. The plasmid pl899 (harboring a genenticin-resistance gene expression cassette; Namiki et al., 2001) was digested with EcoRI, 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 Smal, and the fragment was then ligated into the blunt-ended pl899 vector. The resulting pl899-based plasmid harboring the dsGFP expression cassette was designated pl899-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, pGtiS10, 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. Amplified cDNA fragments were first sub-cloned into the pGEM-T easy vector (Promega), and the resulting plasmids were designated pB2113-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 plB1-GFP (Yaegashi et al., 2007) was cloned into the pBE2113 vector using XbaI and SacI sites, and the resulting plasmid was designated pB2113-mGFP. The empty vector (pBE2113-p35T) and the vector expressing HCPo of the clover yellow vein virus (pB2113-HCPo) 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 pCGFP by the protoplast-mediated polyethylene glycol (PEG)/CaCl₂ 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 RGF. To induce silencing of GFP, RGF was transformed again with pl99-dsGFP, and the resulting strain, resistant to gentamicin and lacking GFP fluorescence, was designated RiGF. W97 was transformed with pl99-dsGFP to generate a strain that expresses only dsRNA of GFP, designated irGF. Fungal colonies were photographed under bright-field illumination using a digital camera (EOS100D; 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 (Brigueti et al., 1998) was done as described previously (Yaegashi et al., 2007). For co-infiltration, agrobacterial suspensions were prepared at OD₆₀₀= 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 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. RNA and LMW-RNA samples were electrophoresed in 1% agarose gels containing 7 M urea, respectively, and the RNA was transferred to nylon membrane (GE Healthcare) by capillary blotting. The RNA was hybridized with DIG-labeled RNA probe of the EGFP sequence (720 nt; accession no. DQ768212) or the mGFP5 sequence (717 nt; accession no. U87973), correspond to 512 aa and 69 aa and S10PN (LPVSVPLNSQDVRPESGSD) and S10PC (VSEGFRSAYWSYVYASHGVC), respectively, and the RNA was transferred to a nylon membrane (GE Healthcare) by capillary blotting. 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:mt 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).

**Acknowledgments**

This work was supported by a Grant-in-Aid for Young Scientists (B) (JSPS Kakenhi Grant Number: 23780045 to H.Y.) and the program for Promotion of Basic and Applied Research for Innovation in Bio-Oriented Industries (to S.K. and H.Y.).

**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.

**References**


Ding, S.W., Voinnet, O., 2007. Antiviral immunity directed by small RNAs. Cell 130, 1155–1158.


Jacob, B.L., Langland, J.O., 1996. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology 219, 339–349.


Kanematsu, S., Sasaki, A., Onoue, M., Oikawa, Y., Ito, T., 2010. Extending the fungal host range of a partitivirus and a mycoreovirus from *Rosellinia necatrix*.