Virology 444 (2013) 409–416

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

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

Hajime Yaegashi [a,*], Nobuyuki Yoshikawa [b], Tsutae Ito [a], Satoko Kanematsu [a]

[a] Apple Research Station, National Institute of Fruit Tree Science, National Agriculture and Food Research Organization (NARO), 92 Shimokuriyagawa, Morioka, Iwate 020-0123, Japan
[b] Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

ARTICLE INFO

Available online 27 July 2013

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

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

* Corresponding author. Fax: +81 19 641 3819.
E-mail address: hyae@affrc.go.jp (H. Yaegashi).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.07.010

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,

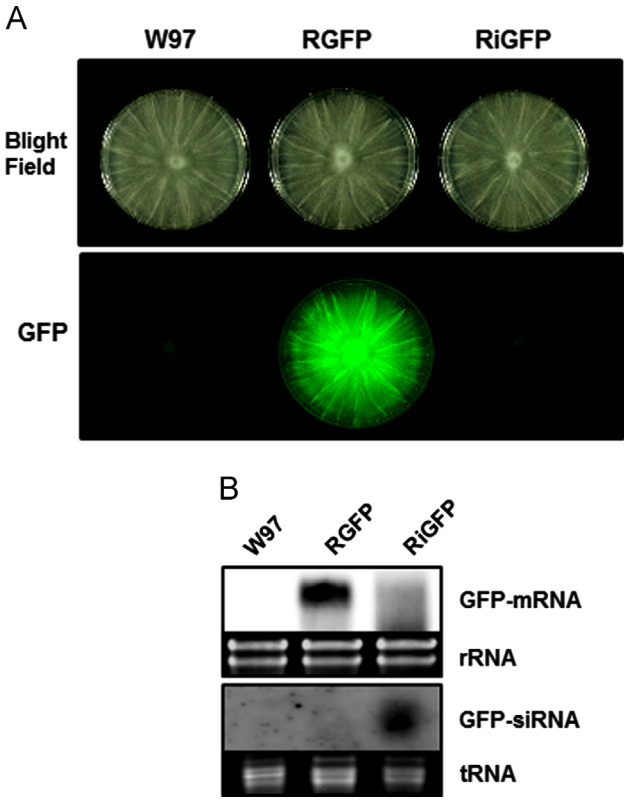

Fig. 1. Generation of *Rosellinia necatrix* GFP-silencing strain. (A) Bright-field (top) and GFP (bottom) images of the parental W97 isolate, GFP-expressing W97 strain (RGFP), and GFP-silencing W97 strain (RiGFP) colonies after 6 days of culturing. (B) Northern blot analysis of GFP-mRNA and siRNA in 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