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# Significantly low level of small RNA accumulation derived from an encapsidated mycovirus with dsRNA genome

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ABSTRACT

The role of RNA silencing as an antiviral defence has been well elucidated in plants and invertebrates, but not in filamentous fungi. We have previously determined the complete genome sequence of Magnaporthe oryzae virus 2 (MoV2), a dsRNA virus that infects the rice blast fungus *Magnaporthe oryzae*. In this study, we detected small interfering RNAs (siRNAs) from both positive- and negative-strand MoV2 viral RNA, suggesting that the RNA silencing machinery in *M. oryzae* functions against the mycovirus. Cloning and characterisation of MoV2 siRNAs indicated that, in MoV2, the ratio of virus-derived siRNAs to total small RNA is significantly lower than that in either plant viruses or Cryphonectria hypovirus 1 (CHV1), another mycovirus. Nevertheless, any MoV2-encoded proteins did not exhibit RNA silencing suppressor activity in both the plant and fungal systems. Our study suggests the existence of a novel viral strategy employed to evade host RNA silencing.



## Introduction

RNA silencing, a mechanism that represses the expression of specific target genes at the posttranscriptional level, has been observed in a broad range of eukaryotes. A common feature of RNA silencing is the processing of structured or double-stranded (ds) RNA into 21–27 nucleotide (nt) small interfering RNAs (siRNAs) by RNase III-like endonucleases termed Dicers (Tomari and Zamore, 2005). In plants and invertebrates, three major lines of evidence indicate that RNA silencing functions as an antiviral defence. First, suppression of host RNA silencing machinery often results in enhanced susceptibility to viral infection. Second, in many cases, the accumulation of virus-derived siRNAs is observed during virus infection. Third, many viruses encode RNA silencing suppressors, which also function as virus virulence factors (Ding and Voinnet, 2007).

Detailed sequence analyses of virus-derived siRNAs have revealed the viral RNA structures from which siRNAs are generated. Previously, siRNAs from positive-sense, single-stranded RNA (ssRNA) viruses had been thought to arise mainly from dsRNA viral replication intermediates (Ahlquist, 2002). However, cloning and sequencing of virus-derived siRNAs in plants infected with ssRNA viruses such as tombusviruses or carmoviruses demonstrated that the siRNAs are predominantly derived from intramolecular hairpins within the positive-strand viral genomic RNA (Ho et al., 2006; Molnar et al., 2005).

Amongst the filamentous fungi, *Neurospora crassa* is widely used as a model organism for the analyses of RNA silencing mechanisms (Fulci and Macino, 2007). As reported in other eukaryotic model organisms, the cellular components of the RNA silencing such as the RNA-dependent RNA polymerase, the Argonaute protein, and two Dicer orthologues, have been characterised in this fungus (Catalanotto et al., 2006). Furthermore, Segers et al. (2007) showed that a Dicer protein of the chestnut blight fungus *Cryphonectria parasitica* helps protect against mycovirus infections, suggesting that RNA silencing in fungi could serve as an antiviral defence mechanism. In support of this hypothesis, the papain-like protease p29 encoded by Cryphonectria hypovirus 1 (CHV1)-EP713 can suppress RNA silencing in both *C. parasitica* and a heterologous plant system (Segers et al., 2006).

Only two studies have demonstrated the presence of mycovirus-derived siRNAs in filamentous fungi; Aspergillus niger virus in *Aspergillus nidulans* and CHV1-EP713 in *C. parasitica* (Hammond et al., 2008; Zhang et al., 2008). In *Aspergillus*, the accumulation level of Aspergillus niger virus-derived siRNA, which was detected by Northern blot analysis, appears to be very low (Hammond et al., 2008). However,

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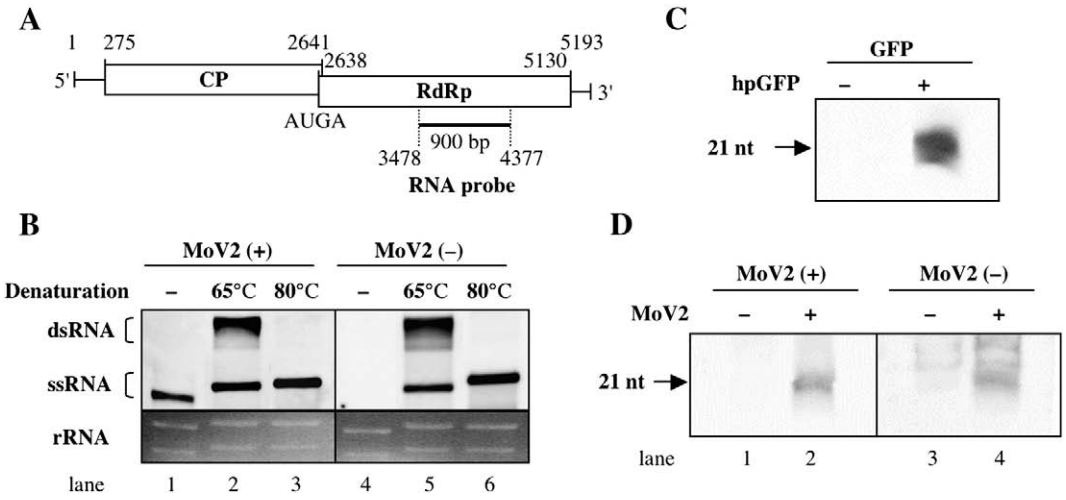


**Fig. 1.** Detection of viral genomic RNA and virus-derived siRNA. (A) Genome organisation of Magnaporthe oryzae virus 2 (MoV2). The viral genome contains two large ORFs overlapping by tetranucleotide AUGA on the same strand, encoding a coat protein (CP) and an RNA dependent-RNA polymerase (RdRp), respectively. Numbers indicate nucleotide positions in the genome. Below the scheme, the region of MoV2 probes used for Northern blot analyses is indicated. (B) Detection of MoV2 genomic RNA. Total RNAs extracted from MoV2-infected strain of *Magnaporthe oryzae* (Ken 60-19) were untreated (lanes 1 and 4), or heat-denatured before electrophoresis at 65 °C (lanes 2 and 5) or 80 °C (lanes 3 and 6) for 10 min. Northern blot analysis was performed using strand-specific probes to detect positive or negative-strand RNAs as indicated at the top of the figure. Ethidium bromide (EtBr) straining of ribosomal RNA (rRNA) was used as the loading control (lower panel). Unheated total RNA migrates faster than heat-denatured one (compare rRNA bands in lanes 1 and 4 with 2 and 5). Positions of dsRNA and ssRNA of MoV2 are indicated on the left. (C) Detection of hpGFP-derived siRNA as a positive control. Low-molecular-weight RNAs (40 μg) extracted from wild-type strain (−) and hpGFP transformant (+) of MoV2-infected strains (Ken 60-19) were separated in a 16% denaturing polyacryl amide gel. The blots were hybridised with a GFP-specific RNA probe. The migration position of the 21 nt microRNA Marker is indicated on the left. (D) Detection of virus-specific small RNAs. Low-molecular-weight RNAs (100 μg) extracted from *M. oryzae* strains were separated in a 16% denaturing polyacryl amide gel: lanes 1 and 3, virus free strain Ken 54-20 (−); lanes 2 and 4, MoV2-infected strain Ken 60-19 (+). Northern blot analysis was performed using strand-specific probes as indicated at the top of the figure, corresponding to panel B. The position of molecular marker is indicated by arrows on the left.

CHV1-EP713 siRNAs accounted for the majority (73%) of the total small RNAs cloned from CHV1-EP713-infected *C. parasitica* (Zhang et al., 2008), which is comparable to the percentage of most plant viruses-derived siRNAs (43–97%; Du et al., 2007; Ho et al., 2006, 2007; Molnar et al., 2005). However, whether mycoviruses are generally attacked by the host RNA silencing remains unclear, and if so, how do they counteract this defence mechanism to establish infection?

The ascomycete fungus *Magnaporthe oryzae* is the causal agent of blast disease, which is the most serious threat to cultivated rice worldwide (Talbot, 2003). The availability of the *M. oryzae* genome sequence (Dean et al., 2005) provides a model system for elucidating the molecular basis of fungal plant diseases. Magnaporthe oryzae virus 2 (MoV2) isolated from *M. oryzae* strain Ken 60-19 is an encapsidated dsRNA virus belonging to the family *Totiviridae* (Maejima et al., 2008). Its dsRNA genome is 5,193 bp and contains two large open reading frames (ORFs) encoding a putative coat protein (CP) and an RNA dependent-RNA polymerase (RdRp) (see Fig. 1A). Although it is known that RNA silencing pathway of *M. oryzae* can be initiated by hairpin-RNA-expressing transgenes or endogenous transposons (Kadotani et al., 2003; Murata et al., 2007), its role in defence against mycoviruses including MoV2 has not been demonstrated yet.

In this study, we characterised MoV2-derived siRNAs to investigate the role of RNA silencing against mycovirus infection in *M. oryzae*. Unexpectedly, the accumulation level of MoV2 siRNAs in the host fungus is extremely low compared to that of CHV1. Moreover, because MoV2-encoded proteins did not display a silencing suppression activity both in heterologous plant system and in host *M. oryzae*, we suggest that MoV2 could evade host RNA silencing by a novel mechanism without a viral RNA silencing suppressor.

## Results

### *Detection of MoV2 viral RNA and virus-derived siRNA*

To examine the nature of MoV2 viral RNA in host cells, we extracted total RNA from Ken 60-19 strain infected with MoV2 and performed Northern blot analyses with both positive- and negative-strand RNA probes corresponding to the RdRp coding region (Fig. 1A). Without heat-denaturation, the positive-strand-specific probe detected a single band, and the negative-strand-specific probe did not detect any bands (Fig. 1B, lanes 1 and 4). When samples were denatured at 65 °C, both positive-and negative-strand-specific probes identified two bands with distinct electrophoretic mobilities (Fig. 1B, lanes 2 and 5). At 80 °C, both probes detected only the faster-migrating band (Fig. 1B, lanes 3 and 6). Since heat-denaturation generally converts duplex RNA into ssRNA as the temperature rises, the slower and the faster bands in lanes 2, 3, 5 and 6 are considered to be MoV2 genomic dsRNA and ssRNA, respectively. Accordingly, RNA detected without heat-denaturation (Fig. 1B, lane 1) proved to be positive-strand ssRNA. It is also assumed that MoV2 dsRNA viral genome could not hybridise with the RNA probes without heat-denaturation (Fig. 1B, lanes 1 and 4). Because the faster-migrating band was not detected in lane 4, negative-strand ssRNA may not be free in the cytoplasm. Taken together, these results suggest that MoV2 viral RNA in host mycelia consists of genomic dsRNA as well as positive-strand RNA, but very little, if any, negative-strand RNA.

Next, to examine whether MoV2 could trigger RNA silencing in *M. oryzae*, we attempted to detect MoV2-derived siRNAs with Northern blot analyses. As a positive control for siRNA detection, a hairpin-GFP RNA-expressing plasmid (phpGFP), which is a potent RNA silencing inducer in *M. oryzae* (Kadotani et al., 2003), was introduced into Ken 60-19 strain (designated as Ken 60-19$^{hpGFP}$). In the low-molecular-weight (LMW) RNA from Ken 60-19$^{hpGFP}$, the accumulation of GFP-derived siRNA was detectable around 21 nt (Fig. 1C). Subsequently, LMW RNAs from Ken 60-19 (MoV2-infected strain) and Ken 54-20 (virus free strain) were extracted and hybridised with MoV2-specific probes (the same probes as shown in Fig. 1B). As a result, MoV2-specific small RNAs were detected at the position of about 21 nt using both positive- and negative-strand-specific RNA probes with similar hybridisation signal intensities, as shown in Fig. 1D. This result suggests that both strands of the MoV2 genome are targeted and processed into siRNAs by the RNA silencing machinery in *M. oryzae*. Remarkably, however, MoV2-derived siRNAs accumulated to a lower degree than hpGFP-derived siRNA (Figs. 1C and D). Indeed, a larger

**Table 1**
List of small RNAs derived from MoV2 genome RNA.

| Small RNA | Sequence (5′ to 3′)[a] | Length (nt) | Orientation [b] | Coordinate[c] |
|---|---|---|---|---|
| si1 | ACACGAACACGAAAUGGCUCA | 21 | + | 2026–2046 |
| si2 | ACACGAAAUGGCUCACGA | 18 | + | 2032–2049 |
| si3 | UCGUACCGAUGAAGACACCGGC | 22 | − | 386–407 |
| si4 | UCGCGUGUUCUGAGCGUGGCGC | 22 | − | 491–512 |
| si5 | UCCGGUCGGUCUCGUCACCC | 20 | − | 2064–2083 |
| si6 | AGCGCCACCGACGUUACCGCCGA | 23 | − | 2316–2338 |
| si7 | UGUGACGUGGCGAUGUUGGA | 20 | − | 3547–3566 |

[a] Seven sequences were identified to match MoV2 genomic RNA by BLAST search from 1370 small RNAs obtained.
[b] "+", siRNA derived from positive-strand RNA of MoV2; "−", siRNA derived from negative-strand.
[c] The loci of MoV2 genome where small RNAs match.

amount of LMW RNA and a longer exposure time were needed to detect MoV2 siRNA. In addition, the sensitivity of MoV2-derived siRNA detection was low but apparent, even when using another probe corresponding to the CP coding region instead of RdRp (data not shown).

### *Cloning and sequencing of MoV2-derived small RNAs*

To characterise in detail the size and the origin of MoV2 siRNAs, we cloned and sequenced small RNAs isolated from the mycelia of MoV2-infected *M. oryzae*. Amongst 1370 small RNA sequences, ranging in length from 11 to 34 nt, only seven small RNAs were identical to the sequence of the MoV2 genome based on a BLAST analysis (Table 1). In this study, we referred to the seven MoV2-associated small RNAs as si1–7. The size distribution of si1–7 was 18–23 nt (Table 1), which is similar in size to CHV1 siRNAs (Zhang et al., 2008). Similar to the known small RNAs of other organisms, MoV2 small RNAs displayed a bias toward A or U nucleotides at their 5′ ends (Table 1; Molnar et al., 2007; Schwarz et al., 2003). These seven sequences did not match any *M. oryzae* genomic or mRNA sequences. Although over 50% of CHV1 siRNAs contain 1- or 2-nt terminal or internal nucleotide mismatches with the viral RNA sequence (Zhang et al., 2008), siRNAs obtained in this study showed perfect matches to MoV2 sequences. Moreover, the proportion of MoV2-derived small RNAs to total cloned small RNAs extracted from *M. oryzae* was 0.5% ($n=1370$), which is much lower than that of CHV1-derived small RNAs to total small RNAs from its host mycelium (73%, $n=233$; Zhang et al., 2008). The small RNAs that were not homologous to MoV2 sequences originated primarily from rRNA (72%; Fig. 2A), similar to findings for small RNA from *C. parasitica* (Zhang et al., 2008). *C. parasitica* small RNAs that were not homologous to CHV1 sequence, however, showed an even distribution and are considered random degradation products (Zhang et al., 2008). In contrast, the small RNA population of *M. oryzae* exhibited a significant peak at 20 nt in length (Fig. 2B), suggesting that non-virus-derived small RNAs are RNA-induced transcriptional silencing (RITS)-associated siRNAs as found in budding yeast (Moazed, 2009; Verdel et al., 2004), or QDE-2-interacting small RNAs (qiRNAs), which originate mostly from the ribosomal DNA locus, as found in *N. crassa* (Lee et al., 2009).

When si1–7 were mapped onto the MoV2 genome RNA (Fig. 2C), both positive and negative polarity were included, which is consistent with the observation that positive- and negative-strand-specific siRNAs were detected with similar signal intensities by Northern blot analysis (Fig. 1D). However, based on the RNA folding algorithm mfold (Zuker, 2003), none of the regions adjacent to these MoV2 siRNAs except si6 were predicted to fold into stable stem–loop structures from which siRNAs could be processed by Dicers (data not shown). These results indicate that the origin of MoV2 siRNAs might be viral dsRNA rather than intramolecular hairpins within viral ssRNA.

### *A quantitative comparison of siRNA accumulation between MoV2 and CHV1*

Since the proportion of MoV2 siRNAs to total cloned small RNAs (0.5%) was at least 150-fold smaller than that of CHV1 siRNAs (73%), we speculated that the accumulation of MoV2 viral RNA in mycelia was significantly lower than that of CHV1. To examine this possibility, we compared the accumulation level of both MoV2 and CHV1 viral RNA in their host mycelia by Northern blot analyses. RNA probes to detect each virus were designed to have the same length (about 900 nt with similar GC content). Both MoV2 and CHV1 viral RNAs were detected with the same exposure time (Figs. 3A and B) when 500 ng of total RNA from respective mycelia was loaded, suggesting that the accumulation of MoV2 viral RNA in *M. oryzae* is comparable to that of CHV1 viral RNA in *C. parasitica*. In accordance with this assumption,

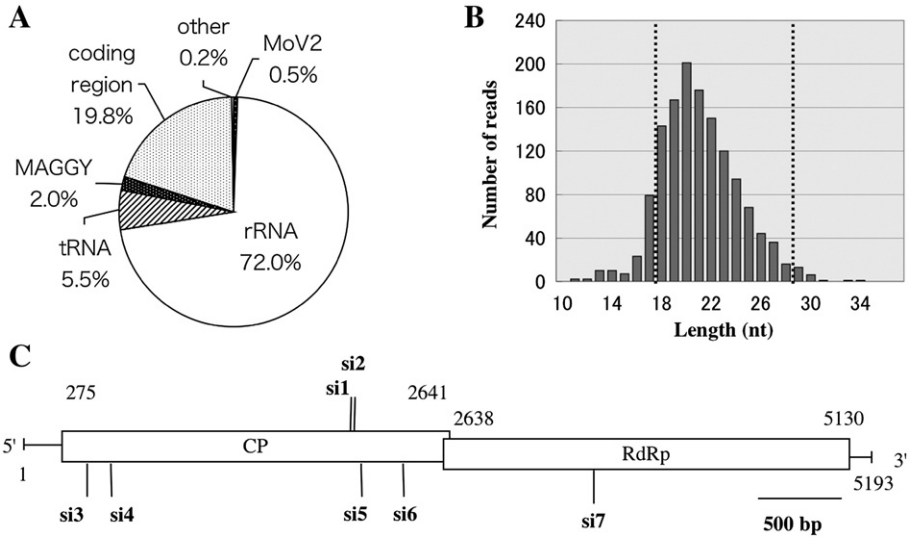


**Fig. 2.** Profiling of small RNAs from MoV2-infected *Magnaporthe oryzae*. (A) Pie chart illustrating percentages for the individual small RNA categories relative to the total number of small RNAs sequenced. Small RNAs were classified into MoV2-assosiated, rRNA-associated, tRNA-associated, MAGGY-associated and protein-coding gene-associated small RNAs. (B) Size distribution of sequenced total small RNAs in MoV2-infected *M. oryzae*. Numbers of sequenced small RNAs with a particular length are shown. The sizes of small RNAs are indicated below the columns. Dotted lines show the range corresponding to the purified-gel which 18–28 nt RNAs were encompassed by the mobility of size marker. (C) Origin and polarity of MoV2-derived small RNAs. The positions where siRNAs were derived are indicated along the 5.2 kbp MoV2 genome RNA. Virus-derived siRNAs oriented from the positive strand are indicated above the diagram, and virus-derived siRNAs oriented from the negative strand are indicated below the diagram.

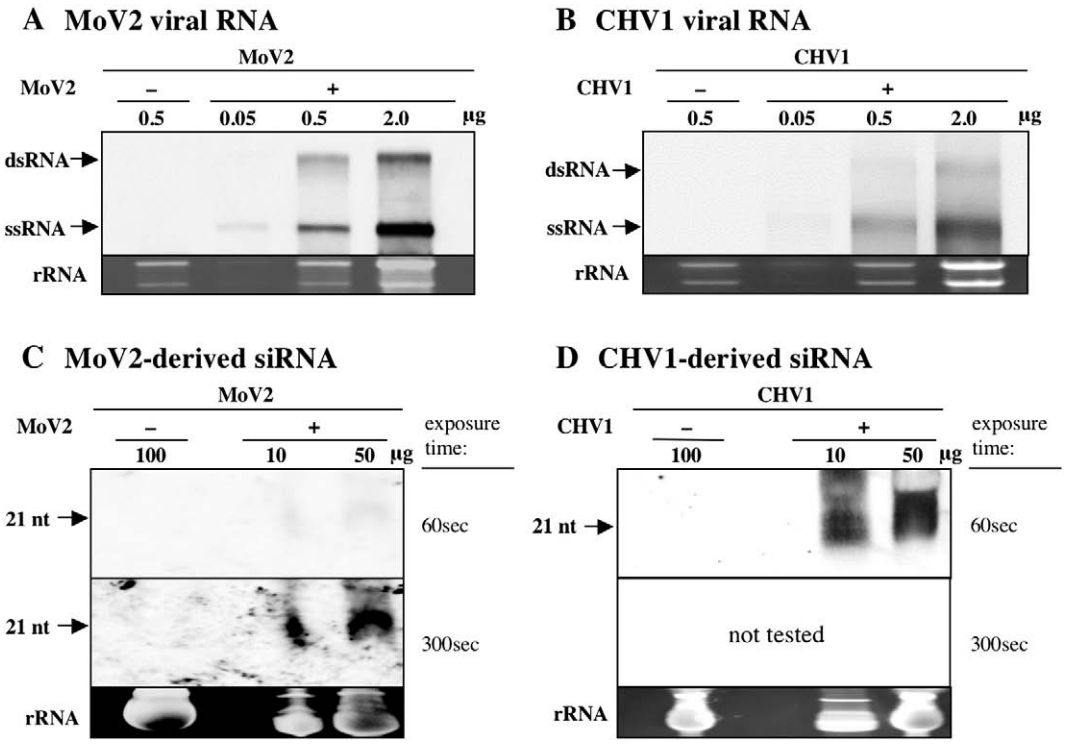


**Fig. 3.** Comparison of the accumulation of siRNA between MoV2 and CHV1. (A, B) Northern blot analysis to compare the amount of MoV2 viral RNA accumulation with that of CHV1 viral RNA accumulation. Total RNAs from virus-free strain Ken 54-20 (−) and MoV2-infected strain Ken 60-19 (+) were loaded in 1.2% agarose gels and hybridised with MoV2 RNA probe (A). Total RNAs from virus-free strain A-1 (−) and CHV1-infected strain EP713 (+) were loaded in 1.2% agarose gels and hybridised with CHV1 RNA probe (B). The amount of RNA loaded is indicated above the panels. Exposure time in both analyses was 5 min. EtBr straining of rRNA was used as the loading control. (C, D) Northern blot analysis to compare the amount of MoV2-derived siRNA with that of CHV1-derived siRNA. Low-molecular-weight RNAs from virus-free strains (−) and mycovirus-infected strains (+) were separated in a 16% denaturing polyacryl amide gels. Panels C and D were detected using the corresponding probes to panels A and B, respectively. Exposure times are indicated at the right side of images and the positions of molecular markers are indicated by arrows on the left. EtBr straining of rRNA was used as the siRNA loading control.

we have confirmed that the concentrations of viral dsRNA in host mycelia are almost equal between MoV2 and CHV1 by using ethidium bromide staining under the same assay conditions (data not shown).

Next, we compared the accumulation level of virus-derived siRNAs between MoV2 and CHV1. We extracted LMW RNAs from each strain (MoV2-infected and -uninfected *M. oryzae*, and CHV1-infected and -uninfected *C. parasitica*) and performed Northern blot analyses with the corresponding probes as in Figs. 3A and B, respectively. CHV1 siRNAs were clearly detected with an exposure time of 60 s; however, MoV2 siRNAs were not detected with a 60-s exposure time, and were detected only after a more prolonged exposure time (300 s; Figs. 3C and D). These observations suggest that MoV2 siRNAs occur at a much lower level than CHV1 siRNAs.

### *Assay for the RNA silencing suppression activity of MoV2-encoded proteins*

Many DNA and RNA viruses encode RNA silencing suppressors to inhibit the host-mediated RNA silencing defence mechanisms. To test whether MoV2-encoded proteins can suppress RNA silencing, we employed the *Agrobacterium* infiltration assay using *Nicotiana benthamiana* plants (Lichner et al., 2003; Voinnet et al., 1999).

*Agrobacterium* strains carrying a vector expressing MoV2 CP or RdRp genes under the control of the cauliflower mosaic virus 35S promoter were co-infiltrated with those carrying 35S-GFP into *N. benthamiana* leaves. HC-Pro and p19, well-known viral RNA silencing suppressors (Brigneti et al., 1998; Kasschau and Carrington, 1998; Silhavy et al., 2002), were used as positive controls. In the patches infiltrated with GFP and GUS (negative control), GFP fluorescence was observed 2–3 days postinoculation (dpi), followed by a decrease and then disappearance of fluorescence due to RNA silencing of the GFP gene (Figs. 4, 5 dpi). In contrast, leaves co-infiltrated with GFP and HC-Pro or p19 remained bright green at 5 dpi due to suppression of GFP silencing (Fig. 4). Patches expressing MoV2 CP and RdRp showed no obvious GFP fluorescence at 5 dpi (Fig. 4), indicating that MoV2-encoded proteins have no silencing suppression activity in this plant system.

### *Assay for the RNA silencing suppression activity of MoV2 in M. oryzae*

The LTR-retrotransposon MAGGY induces siRNA-mediated RNA silencing in *M. oryzae* (Murata et al., 2007). The dicer-gene knockout

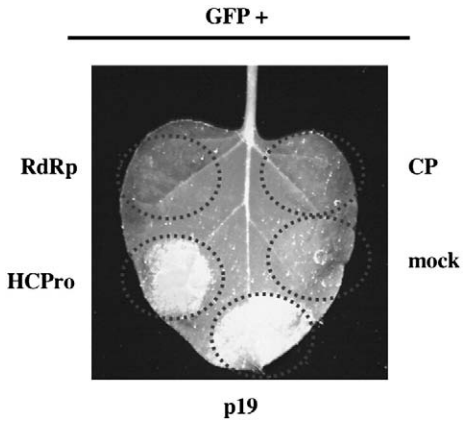


**Fig. 4.** RNA silencing suppressor assay of MoV2-coding genes. *Nicotiana benthamiana* leaves were co-infiltrated with *Agrobacterium* strain expressing GFP together with indicated proteins. GFP fluorescence was imaged under UV light at 5 days postinoculation. GFP fluorescence can be observed as white.

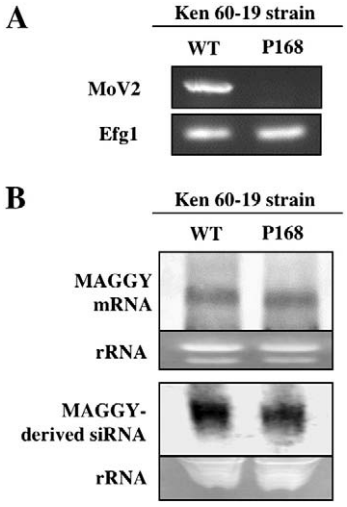


**Fig. 5.** RNA silencing suppressor assay of MoV2 in *Magnaporthe oryzae*. (A) Production of virus-free isolate of *M. oryzae* Ken 60-19 strain. RNAs extracted from the Ken 60-19 wild-type and the progeny-168 (P168) isolate were analysed by RT-PCR to detect the viral RNA. *M. oryzae* transcription factor gene (Efg1) was used for positive control. (B) Northern blot analysis to detect MAGGY mRNA and MAGGY-derived siRNA. Total RNAs (4 μg) for mRNA detection and LMW RNA (50 μg) for siRNA detection were hybridised with MAGGY-specific RNA probe. EtBr straining of rRNA was used as the loading control.

mutant exhibits a significant increase in the accumulation of MAGGY mRNA, indicating that the RNA silencing machinery effectively represses the expression of MAGGY genes in *M. oryzae* (Murata et al., 2007). In accordance with this finding, our sequence analysis revealed the presence of MAGGY-derived small RNA in the MoV2-infected strain Ken 60-19 (Fig. 2A). Therefore, if MoV2 can suppress host RNA silencing pathways, MAGGY transcript levels might be upregulated by MoV2 infection.

To test this postulate, we first obtained a MoV2-free isolate from the wild-type Ken 60-19 by single conidia isolation. Conidia grown on an oatmeal agar plate were dropped onto a water agar plate by a gentle tap and were isolated individually under a light microscope. Virus-free progeny were screened by RT-PCR analysis and an isolate referred to as progeny-168 (P168) was obtained (Fig. 5A). Thereafter, to compare the accumulation levels of MAGGY mRNA and MAGGY-derived siRNA between Ken 60-19 wild type and P168, we performed Northern blot analyses using a MAGGY-specific RNA probe. No significant differences were observed between the wild type and P168 isolates in either mRNA or siRNA accumulation (Fig. 5B). These results suggested that MoV2 could not suppress RNA silencing in *M. oryzae*.

## Discussion

In this study, we found that the accumulation of MoV2 siRNA in *M. oryzae* is significantly lower than that of CHV1 siRNA in *C. parasitica* based on two different experiments; sequencing of small RNAs cloned from MoV2-infected *M. oryzae* (Table 1, Fig. 2) and quantitative Northern blot analyses (Fig. 3). Our results suggest that RNA silencing against MoV2 in *M. oryzae* is apparently less effective as compared with that against CHV1 in *C. parasitica*.

The observed difference did not seem to arise from differences in the accumulation levels of viral RNA between MoV2 and CHV1 (Fig. 3). In addition, we found no defect in the RNA silencing response in MoV2-infected *M. oryzae*, since we detected strong hpGFP- and MAGGY-derived siRNA signals in Northern blot analyses (Figs. 1C and 5B). Thus, we hypothesised that MoV2 would encode a more potent RNA silencing suppressor than the CHV1-encoded p29 protein. However, MoV2-encoded proteins did not function as silencing suppressors in either a well-established plant system (Fig. 4) or in the natural host *M. oryzae* (Fig. 5). This result is consistent with the finding that neither Aspergillus virus 178 nor Aspergillus virus 341, which is closely related to MoV2, suppresses inverted repeat transgene-induced RNA silencing in their hosts (Hammond et al., 2008).

Furthermore, MoV2 viral RNA was less susceptible to RNA silencing than MAGGY mRNA in the same MoV2-infected strain; the number of MoV2-derived siRNAs (7 sequences) was about 4-fold less than that of MAGGY-derived siRNAs (26 sequences; Fig. 2A), although Northern blot analysis indicated that the accumulation level of MoV2 RNA was much higher than MAGGY mRNA (Figs. 1B and 5B). Collectively, MoV2 is highly insusceptible to host RNA silencing and MoV2 encoded proteins did not exhibit RNA silencing suppressor activity.

Except for the silencing suppression by virus-encoded suppressor proteins, the mechanism by which viruses evade RNA silencing has not been described in detail. However, several reports have raised the intriguing possibility that dsRNA viruses might avoid antivirus defence mechanisms by retaining their genomes and RNA synthesising enzymes within their virions (Hammond et al., 2008; Mertens, 2004). In the family *Totiviridae*, including MoV2, studies on Saccharomyces cerevisiae virus L-A (ScV-L-A), the type species of the genus *Totivirus*, have demonstrated that synthesis of both RNA strands occurs within the viral particles (Wickner, 1996). In our study, free MoV2 negative-strand RNA in the host cytoplasm was rare (Fig. 1B), suggesting that MoV2 negative-strand RNA exists only within the virion as a template for viral positive-strand RNA synthesis and that MoV2 also synthesises viral RNA within the virion.

In contrast, CHV1 belonging to the family *Hypoviridae* does not have any viral particles. In addition, plant ssRNA viruses mentioned in the introduction, in which siRNA accumulates as much as CHV1 siRNA in their hosts (43–97%; Du et al., 2007; Ho et al., 2006, 2007; Molnar et al., 2005; Zhang et al., 2008), have viral capsid structures but replicate their genomes in the host cytoplasm after uncoating. Thus, plant viruses and CHV1 expose their genomes to the host cell cytoplasm, which may activate host RNA silencing machinery and be targeted by it.

Recent phylogenetic analyses suggest that dsRNA-mediated RNA silencing machinery was present in the last common ancestor of eukaryotes (Cerutti and Casas-Mollano, 2006; Ullu et al., 2004). This means that dsRNA viruses may have acquired resistance to RNA silencing at very early stages in their evolution. Taken together, it is considered that replicating within the rigid virion structure may be one of the basic strategies for dsRNA viruses to evade host RNA silencing machinery. However, there may be some variations in the interaction between dsRNA mycoviruses and their host. Indeed, suppressor proteins have been identified in some encapsidated dsRNA viruses in the family *Reoviridae*: the σ3 protein encoded by a member of the genus *Orthoreovirus* (Lichner et al., 2003) and the Pns10 protein encoded by a member of the genus *Phytoreovirus* (Cao et al., 2005). Noteworthy, two suppressor proteins, σ3 and Pns10, are not homologues and viruses of the genus *Rotavirus* in the family *Reoviridae* are thought to have no silencing suppressor (Arias et al., 2004). Therefore, these two suppressors may have evolved independently through adaptations to their hosts. In addition, it is known that a dsRNA mycoreovirus is susceptible to RNA silencing antiviral defence response (Segers et al., 2007). Collectively, encapsidated dsRNA viruses may have a large variability in the degree of the protective efficacy of viral particles from RNA silencing depending on the virus-host interactions. Since MoV2 is highly insusceptible to host RNA silencing and is the first example, to our knowledge, of encapsidated dsRNA viruses whose siRNA are cloned and sequenced, further studies on encapsidated dsRNA viruses will reveal the interaction of the viral strategy to counteract with host RNA silencing.

## Materials and methods

### *Fungal strains and growth conditions*

*M. oryzae* strains Ken 60-19 (MAFF 305470) and Ken 54-20 (MAFF 101508), and *C. parasitica* strains EP713 (ATCC 52571) and A-1 (MAFF 410726) were used. These were maintained on PDA agar (Oxoid) at 25 °C. For DNA and RNA extraction, mycelia were cultured in YG medium (0.5% yeast extract, 2% glucose) at 25 °C for 4 to 5 days.

### *RNA purification and Northern blot analysis*

Total RNA was extracted from mycelia using Sepasol–RNA I (Nacalai Tesque, Inc.) according to the manufacturer's protocol. Low-molecular-weight (LMW) RNA fractions were prepared as described previously (Senshu et al., 2009).

Northern blot analysis was performed using Roche's DIG Northern kit. To detect viral RNA or mRNA total RNA was denatured at 65 °C for 5 min and separated on 1.2% agarose gels. To detect siRNAs, LMW RNAs were separated on 16% polyacrylamide, 7 M urea gels. After blotting, the membranes were hybridised with appropriate probes at 42 °C overnight. The DIG labelled RNA probes were prepared with a DIG Northern Starter Kit (Roche, Inc.) following the manufacturer's protocol. For the DNA template corresponding to the MoV2 probe, MoV2 cDNA (Maejima et al., 2008) was used. The DIG labelled RNA probe used to detect CHV1 and MAGGY sequences were generated as described previously (Zhang et al., 2008; Murata et al., 2007). The microRNA Marker (New England Bio-Labs, Inc.) was used for the determination of small RNA sizes. The detection and analysis of viral RNAs and siRNAs were performed with LAS 4000 mini (Fujifilm) and Multi Gauge software version 3.0 (Fujifilm).

### *Hairpin GFP vector plasmid construction and transformation*

The sense and antisense segments of the GFP sequence were amplified from a DsGFP-DNA construct (Takahashi et al., 2006) and cloned into pBF101 under the control of the *trp*C promoter (Kimura et al., 1994). The cutinase intron (Nakayashiki et al., 2005) was amplified from the genome of *M. oryzae* (Ken 54-20) with the primers: cut-int-F (5′-GAA GAT CTG ATA TCG CTG GAG GAT ACA GGT GAG C-3′) and cut-int-R (5′-GAA GAT CTG ATA TCG CCG TTC CCT GGC TGT GTG TT-3′). The intron PCR fragment was inserted between the sense and antisense GFP genes by inverse PCR at *Bgl*II (phpGFP). The resulting plasmid phpGFP was co-transformed with pSH75, which contains a hygromycin-resistant gene cassette (Kimura and Tsuge, 1993), into *M. oryzae* as described previously (Sweigard et al., 1995). Transformants were selected on growth medium containing 300 μg/ml Hygromycin B (Wako). Successful transformation was confirmed by Southern hybridisation using a DIG application Kit (Roche, Inc.). Fungal genomic DNA was extracted using the CTAB procedure (Mori et al., 2008), and was digested with the appropriate restriction enzymes.

### *Cloning and sequencing of virus-derived siRNAs*

Cloning of small RNAs was performed with a miRNA Cloning Kit (Dyna Express) following the manufacturer's protocol. The cloned cDNAs from small RNAs were sequenced by DNA sequencer (ABI PRISM3130 DNA Sequencer). BLAST searches were performed using the Broad Institute database downloaded from NCBI (http://www.ncbi.nlm.nih.gov/).

### *Suppressor assays*

MoV2 CP and RdRp coding regions were cloned into pENTA, a pUC19-based plasmid containing the attL1-MCS-attL2 region of pENTR1A (Invitrogen). Then, MoV2 CP and RdRp fragments were subcloned into the pEarleyGate 100 vector (Earley et al., 2006) with Gateway LR Clonase II Enzyme Mix (Invitrogen). GFP, HcPro and p19 constructs were described previously (Senshu et al., 2009). pCAMBIA 1301 was used as an empty vector for mock inoculation. Agroinfiltration and observation were performed as described previously (Senshu et al., 2009).

### *Isolation of a virus free progeny*

For single conidium isolation, conidia were harvested from Ken 60-19 strain grown on oatmeal cultures (Mori et al., 2008). RT-PCR was performed with total RNA using AMV Reverse Transcriptase XL and rTaq (TaKaRa Bio, Inc.) according to the manufacturer's protocol. To detect MoV2, primers MoV-RdRp-F and MoV2-RdRp-R were used. As an endogenous control, the *M. oryzae* transcription factor gene Efg1 was amplified with primers: JF186 (5′-ATG TAC CAC AGC AGC CAC AAG AAG CAA GCC C-3′) and JF187 (5′-CCA TGT GCC TTG ATA CTT GCC ATA ACC TCC-3′).

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