Establishment of *Neurospora crassa* as a model organism for fungal virology

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The filamentous fungus *Neurospora crassa* is used as a model organism for genetics, developmental biology and molecular biology. Remarkably, it is not known to host or to be susceptible to infection with any viruses. Here, we identify diverse RNA viruses in *N. crassa* and other *Neurospora* species, and show that *N. crassa* supports the replication of these viruses as well as some viruses from other fungi. Several encapsidated double-stranded RNA viruses and capsid-less positive-sense single-stranded RNA viruses can be experimentally introduced into *N. crassa* protoplasts or spheroplasts. This allowed us to examine viral replication and RNAi-mediated antiviral responses in this organism. We show that viral infection upregulates the transcription of RNAi components, and that Dicer proteins (DCL-1, DCL-2) and an Argonaute (QDE-2) participate in suppression of viral replication. Our study thus establishes *N. crassa* as a model system for the study of host-virus interactions.
Fungal viruses or mycoviruses are omnipresent in all major groups of fungi, and the majority of them show asymptomatic and frequently mixed infections. Like viruses of other host kingdoms, fungal viruses have different types of genomes, although they largely are double-stranded (ds) and single-stranded (ss) positive-sense (+) RNA viruses. Mycoviruses are increasingly discovered in fungi by the conventional detection of dsRNAs, a sign of RNA virus infection, via high-throughput sequencing of transcripts and/or dsRNA, and in silico mining of publicly available transcriptomic data. Fungal viruses commonly have no extracellular route for entry with the exception of an encapsidated ssDNA virus, and are generally difficult to experimentally introduce into their host fungi extracellularly, which often impedes the progress of this kind of study. Only for some mycoviruses have protocols been developed for the experimental introduction or inoculation. Virion transfection is frequently used for encapsidated dsRNA viruses such as reoviruses, toiviruses, megabirnaviruses, and partitiviruses. For several capsidless (+)ssRNA viruses, transfection with in vitro-synthesized full-length genomic RNA or transformation with full-length cDNA are available. Protoplast fusion-based virus horizontal transmission was shown to be useful between virus-infected donors and many recipients for any viruses, i.e., encapsidated and capsidless viruses.

**Neurospora crassa** (family Sordariaceae) is a filamentous ascomycete used as a research material for the one-gene–one-enzyme hypothesis by Beadle and Tatum in 1941. This fungus has served as a model eukaryotic multicellular organism for genetics, developmental biology, and molecular biology. In particular, circadian rhythm-based physiological regulation, RNA interference (RNAi) post-transcriptional gene silencing, and DNA methylation-mediated epigenetic control have been pioneered by researchers using *N. crassa*. The advantages of this fungus over other organisms, especially over other filamentous fungi, include the public availability of a number of biological and molecular tools, biological tractability, i.e., rapid vegetative growth and ease of sexual mating, shared techniques, a well-annotated genome sequence, and single-gene knockout (KO) collection available from the Fungal Genetics Stock Center (FGSC) (http://www.fgsc.net). Surprisingly, despite these merits, this fungus is not utilized in virological studies.

RNAi, also known as RNA silencing, occurs in a wide variety of eukaryotic organisms, including animals and plants. The basic process of RNAi involves dsRNA that is recognized and diced by Dicer into small RNAs. These small RNAs then are incorporated into the effector Argonaute complex for repression of the target gene expression at transcriptional and post-transcriptional levels. RNAi pathways in fungi were deciphered for the first time in *N. crassa*. The known pathways in *N. crassa* operate at the post-transcriptional level, and are largely divided into two groups: meiotic and mitotic RNAi depending on which stage RNAi occurs in. The second group is further categorized into two: quelling and qRNAi (QDE-2-interacting small RNA)-mediated silencing depending on how dsRNA is generated. *N. crassa* has three RNA-dependent RNA polymerase (RDR), two Dicer (Dicer-like DCL), and two Argonaute (Argonaute-like AGL) proteins. Two vegetative RNAi pathways share players, while aberrant RNA is generated from repetitive chromosomal regions and the UV-damaged nuclear genome, respectively. Although RNAi in some fungi has been shown to act as an antiviral defense-system response as in the case of animals and plants, its role in antiviral response has not been demonstrated in *N. crassa*. Choudhary et al. demonstrated the transcriptional induction of RNAi pathway and other putative antiviral genes by transgenic expression of dsRNA, suggesting that the RNAi pathway may act as part of the viral defense mechanism in this fungus. In this sense, a phytopathogenic ascomycete, *Cryphonectria parasitica* (chestnut blight fungus, family *Cryphonectriaceae*), has been used to dissect antiviral RNAi in the past decade. Of four RDRs, two Dicer, and four Argonaute genes, one Dicer (*dcl2*) and one Argonaute (*agl2*) gene play major roles in antiviral RNAi. Of note is that the key genes are transcriptionally upregulated upon virus infection, for which a general transcriptional activator SAGA (Spt–Ada–Gcn5 acetyltransferase) complex and DCL2 (positive-feedback player) are essential.

Here, we demonstrate the detection of diverse RNA viruses from *N. crassa* and two other *Neurospora* species *N. intermedia* and *N. discreta*, and experimental introduction into the *N. crassa* standard strain of several viruses originally isolated from other fungi using three different methods. Utilizing the biological resources and molecular tools of *N. crassa* led to interesting insights into how virus/host interactions, particularly antiviral RNAi/viral counter-RNAi, are regulated. Collectively, this study establishes a foundation for the study of virology in the model organism *N. crassa*.

**Results**

**Diverse RNA viruses in Neurospora spp.** To elucidate virus infection in wild *Neurospora* species, we first chose six wild *N. intermedia* isolates from Bogor, Indonesia (FGSC strains #2558, #2559, #5098, #5099, #5643, and #5644) and performed a small-scale screen by a conventional method. The presence of dsRNA of ~8 kbp and 5 kbp was observed in the FGSC #2559 and FGSC #5099, respectively (Fig. 1a). The complete sequence of a dsRNA segment from FGSC #2559 determined by next-generation and Sanger sequencing was shown to represent the replicative dsRNA form of a capsidless (+)ssRNA virus, termed Neurospora intermedia fusarivirus 1 (NfFv1, a putative member of the proposed family “Fusariviridae”) (DDBJ/EMBL/GenBank accession #: LC530174). The dsRNA of ~5.0 kbp in FGSC #5099 has yet to be characterized.

Mining of virus sequences from transcriptomic data has been a trend for hunting and discoveries of viruses. Prompting us to mine for virus sequences infecting this fungus. In total, six different types of RNA viruses, specifically four (+)ssRNA viruses (e.g., fusarivirus, deltaflexivirus, alpha-like virus, and ourmia-like virus), one dsRNA virus (partitivirus), and one uncategorized RNA virus, were detectable in each data set for 15 out of 134 different *N. crassa* field-collected strains (Table 1 and Supplementary Table 1). To confirm the virus-like sequences in vivo, we detected dsRNAs from 12 out of 15 strains (Fig. 1b). We also obtained 16 well-assembled fragments of fusariviruses divided into three subgroups (namely A, B, and C in clade I) from 12 fungal strains of different geographical origins and found that 7 fungal strains were infected by a single fusarivirus and 5 other strains were infected by two fusariviruses (Table 1). These suggest a widespread nature of fusariviruses (see below) in members of the genus *Neurospora* of different geographical origins (Table 1, Fig. 2, and Supplementary Fig. 1). Among them, a single fusarivirus (subgroup A) harbored in the Louisiana isolate JW60, closest to the standard *N. crassa* strain 74-OR23-1VA, was fully determined by Sanger sequencing as the standard *N. crassa* fusarivirus (DDBJ/EMBL/GenBank accession #: LC530175), termed Neurospora crassa fusarivirus 1 (NfFv1) (Fig. 2a). NfFv1-JW60 was used in the subsequent investigation into virus/host interactions, because the standard *N. crassa* strain singly infected by this virus could readily be obtained. Similarly, we completely sequenced the genomic segments, dsRNA1 and dsRNA2, of a partitivirus termed Neurospora crassa partitivirus 1
(NcPV1) from the wild Floridan isolate JW35 (DDBJ/EMBL/GenBank accession #: LC530176 and LC530177) (Fig. 3a). *Neurospora discreta* strain FGSC #8579 (Belen, New Mexico, USA), which is mostly used as a standard strain, has recently been suggested to carry a fusarivirus, *Neurospora discreta* fusarivirus 1 (NdFV1), *Neurospora crassa* fusarivirus 1 (NcFV1), and *Neurospora crassa* partitivirus 1 (NcPV1). Multiple dsRNA bands were detected in *N. discreta* isolate FGSC #8579 that is expected to represent the genomes of several RNA viruses (Table 1). The 15 field-collected *N. crassa* strains were subjected to dsRNA analyses together with a potential virus-free standard strain FGSC #2489 (Supplementary Table 1). The discrete dsRNAs of 2–3 kbp detectable in *N. crassa* JW45, JW70, and D23 may be defective RNAs.

Sequence and phylogenetic analyses of the characterized fusariviruses of *Neurospora* spp. showed that (1) viral RdRP was more conserved than the P2 protein (RdRP: 29.94–99.33%, P2: 14.96–99.48%), (2) NcFV1, NiFV1, and NdFV2 belong to the same clade (I) and share larger genome size, while NdFV1 contains a coiled-coil domain in P2 and belongs to another clade (II), and (3) *N. crassa* fusariviruses appeared to be separated into three subgroups, NcFV1A, B, and C in clade I, based on the phylogenetic relationship and amino acid sequence identity of RdRP and P2 (Fig. 2). All five *Neurospora* partitiviruses, NcPV1

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**Fig. 1 Virus detection in Neurospora spp. and experimental introduction of heterologous viruses into the Neurospora crassa strain.** Agarose gel electrophoresis of dsRNA fractions obtained from *N. intermedia* (a), *N. crassa* (b), and *N. discreta* (c). After electrophoresis, dsRNA extracted from isolates of *Neurospora* spp. shown on the top of each gel was stained with GelGreen (Biotium, Inc.). Fully sequenced viruses are *Neurospora intermedia* fusarivirus 1 (NiFV1), *Neurospora crassa* fusarivirus 1 (NcFV1), and *Neurospora crassa* partitivirus 1 (NcPV1). Multiple dsRNA bands were detected in *N. discreta* isolate FGSC #8549 that is expected to represent the genomes of several RNA viruses (Table 1). The 15 field-collected *N. crassa* strains were subjected to dsRNA analyses together with a potential virus-free standard strain FGSC #2489 (Supplementary Table 1). The discrete dsRNAs of 2–3 kbp detectable in *N. crassa* JW45, JW70, and D23 may be defective RNAs.

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and NdPV1–4, share molecular attributes with other known partitiviruses; dsRNA1 encodes RdRP and dsRNA2 encodes CP (Fig. 3a, b). They have all been shown to belong to the genus Partitiviridae: 

**Table 1 List of Neurospora viruses and their host fungal strains.**

| FGSC #  | Strain name | Geographic origin | Mating type | SRA file | Virus (virus abbreviation: accession no., subgroup type) |
|---------|-------------|-------------------|-------------|----------|--------------------------------------------------------|
| 2489    | 74-OR23-1VA | Marrero, Louisiana, U.S.A. | A          | SRR797950 | Virus free (a standard, wild-type strain) |
| 3975    | JW35        | Florida, U.S.A.     | a           | SRR08983  | Partitivirus (NcPV1:LC530176, LC530177) |
| 4713    | JW45        | Merger, Haiti       | A           | SRR089840 | Fusivirus (NcPV1:LC586023, B type), alpha-like virus/P3437 |
| 10651   | JW204       | Bayou Chicot, LA, U.S.A. | A          | SRR798021 | Fusivirus (NcFV1, A and B types) |
| 10658   | JW222       | Coon, LA, U.S.A.    | a           | SRR798029 | Fusivirus (NcFV1:LC586028, C type) |
| 10659   | JW224       | Coon, LA, U.S.A.    | a           | SRR798030 | Fusivirus (NcFV1, C type) |
| 10899   | JW164       | Marrero, LA, U.S.A.  | a           | SRR797998 | Fusivirus (NcFV1:LC586025, A and B types), alpha-like virus |
| 10913   | JW190       | Elizabeth, LA, U.S.A. | A           | SRR798014 | Fusivirus (NcFV1:LC586026, A and B types) |
| 10948   | JW27        | Bayou Chicot, LA, U.S.A. | A          | SRR798051 | Fusivirus (NcFV1:LC586022, C type) |
| 10949   | JW59        | Coon, LA, U.S.A.    | a           | SRR798053 | Fusivirus (NcFV1:LC586024, A and C types) |
| 10950   | JW60        | Coon, LA, U.S.A.    | a           | SRR798054 | Fusivirus (NcFV1:LC530175, A type) |
| 10951   | JW70        | Coon, LA, U.S.A.    | A           | SRR798057 | Fusivirus (NcFV1, A and B types) |
| 10983   | JW193       | Elizabeth, LA, U.S.A. | a           | SRR798015 | Fusivirus (NcFV1:LC586027, B type) |
| 10912   | JW188       | Elizabeth, LA, U.S.A. | A           | SRR798013 | Fusivirus (NcFV1), ourmia-like virus |
| 4716    | JW49        | Puilboreau Mt., Haiti | A           | SRR089843 | Ourmia-like virus |
| 8783    | D23         | Florida, U.S.A.     | A           | SRR089764 | Deltafexivirus, alpha-like viruses |
| 2558    | H2121       | Bogor Pasar, Indonesi a | A          | n.a.      | Not detected |
| 2559    | HC2125-1    | Bogor Pasar, Indonesia | a         | DRR248874 | Fusivirus (NiFV1:LC530174) |
| 5098    | H2156       | Pasar Balur, Indonesia | A         | n.a.      | Not detected |
| 5099    | H2158       | Pasar Balur, Indonesia | a         | n.a.      | Unknown virus-like agent (~5.0 kbp) |
| 5643    | P0151       | Bogor, Indonesia    | A           | n.a.      | Not detected |
| 5644    | P0153       | Bogor-4, Indonesia  | A           | n.a.      | Not detected |
| 8579    | W683        | Belen, New Mexico, U.S. | A, a  | SRR1539773 | Fusiviruses (NdFV1, 2), partitiviruses (NdPV1-4), mitoviruses, and endornavirus |
| 8578    | W682        | Belen, New Mexico, U.S. | a, a  | n.a.      | Partitiviruses |

NiFV1 Neurospora crassa fusarivirus 1, NiFV1 Neurospora intermedia fusarivirus 1, NiFV1, 2 Neurospora discreta fusarivirus 1, 2 (LC586029, LC586030), NcPV1 Neurospora crassa partitivirus 1, NdPV1–4 Neurospora discreta partitivirus 1–4 (LC586031-LC586038), n.a. not available.

**Experimental introduction of heterologous viruses into N. crassa.** To elucidate whether N. crassa is able to support the replication of heterologous viruses originated from other fungal families, we chose two relatively well-characterized dsRNA viruses: Rosellinia necatrix partitivirus 2 (RnPV2, genus Alphapartitivirus) able to replicate in N. crassa43,44,45 isolated from the white root rot fungus (Rosellinia necatrix, family Xylariaceae) and mycoviruses 1 (MyRV1, genus Mycoreovirus, family Reoviridae)44,45 isolated from the chestnut blight fungus (C. parasitica). Using purified preparation, the two viruses were transected into spheroplasts of the standard strain of N. crassa. Figure 1d shows the dsRNA profiles of representative colonies infected by the respective virus. Specific genomic dsRNA bands were observed: dsRNA1 and dsRNA2 for RnPV2, and S1–S11 for MyRV1.

An interesting difference was noted; the ratio of dsRNA1:dsRNA2 was smaller in the natural R. necatrix host than in the newly expanded N. crassa host. This is not so surprising for multisegmented, multiparticulate viruses such as partitiviruses in which each genomic segment is packaged separately. This is different from reoviruses that are multisegmented, monoparticulate viruses in which a set of the multiple genomic segments are packaged in a single particle. Indeed, similar phenomena were observed earlier in natural and experimental host fungi after virion transfection. In contrast, the MyRV1 pattern was indistinguishable from the original infection of C. parasitica. It should be noted that, unlike RnPV2, MyRV1 showed unstable infection during the subculture of transfected colonies.

For capsidless viruses, we previously established protoplast fusion techniques for virus introduction, because virion transfection was not applicable. This method was tested for the putative capsidless RNA virus NiFV1 (a fusarivirus) originally hosted by N. intermedia (Fig. 1a). Protoplasts were prepared from the donor strain FGSC #2559 of N. intermedia and the recipient strain of N. crassa (the standard strain 74-OR23-1VA) that harbored a nourseothricin (NTC)-resistance gene. After protoplast fusion, recipient colonies were selected on potato-dextrose agar (PDA) supplemented with NTC (PDA–NTC). Several of them tested positive for NiFV1 and repeatedly anastomosed with the virus-free, NAT-resistant strain of N. crassa. The genetic background of NiFV1-infected recipients was shown to be N. crassa.
crassa by the observation that the recipient could anastomose with the original isogenic recipient strain. Interestingly, NiFV1 dsRNA-replicative form accumulated much less in N. crassa than in the original host, N. intermedia when normalized to starting mycelia (Fig. 1e). NiFV1 appears not to be adjusted well to N. crassa. Similar phenomena were observed when virus contents were compared between their original and experimental host fungi.

In summary, Neurospora spp. was shown to host a variety of viruses that belong to at least six RNA virus families and an unclassified RNA virus group: Fusariviridae, Partitiviridae, Endornaviridae, Reoviridae, Deltaflexiviridae, Narnaviridae, and an unclassified RNA virus group (alpha-like viruses). Among these, fusariviruses appeared to prevail in Neurospora spp. (Table 1 and Supplementary Fig. 1).

Two Dicers and one Argonaut play a major role in antiviral defense during the vegetative phase in N. crassa. Among Neurospora viruses, fully sequenced NcFV1 and NcPV1 were horizontally transferred between vegetatively compatible and incompatible strains of N. crassa. The two viruses were first moved to the N. crassa helper-5 strain (FGSC #8747; Δmat his-3 tk+ hph cyh-1, Bml pan-2) from the original host strains, NcFV1-infected FGSC #10950 (JW60), and NcPV1-infected FGSC #3975 (JW35), by coculture. The two viruses were moved to the N. crassa helper-5 strain has been used for for-ming and resolving heterokaryons (Supplementary Fig. 2). The two viruses were moved to the N. crassa helper-5 strain has been used for for-ming and resolving heterokaryons (Supplementary Fig. 2).
Hereafter, these three viruses were largely utilized in subsequent deeper analyses of virus/host interactions.

We tested for enhancement of NcFV1, NcPV1, and RnPV2 accumulation a total of 14 single- and two double- and one-triple deletion mutant of the standard *N. crassa* strain with deletions of genes related to two RNAi pathways, mitotic silencing (quelling: *qde-1, qde-2, qde-3, dcl-1, dcl-2, qip, and rpa-3*) in the vegetative stage and meiotic silencing by unpaired DNA (MSUD: *Sad-1, Sad-2, sad-3, Sad-4, and sad-5, dcl-1, qip, and sms-2*) 34, or other function (Supplementary Table 2). To this end, we quantitatively compared viral dsRNA and ssRNA by aagarose gel electrophoresis of dsRNA and RT-qPCR of total RNA fractions, respectively, and found that they exhibited similar accumulation profiles in the *N. crassa* mutant strains (Fig. 4a and Supplementary Figure 3). The single-deletion mutants tested, only *Δqde-2* showed ~10-fold and ~25-fold elevation in accumulation of NcFV1 dsRNA-replicative form and viral ssRNA, relative to the wild-type strain, respectively (Fig. 4a). The two dicer genes were anticipated to work redundantly in antiviral defense as in the case for transgene silencing (quelling) 30. To test this hypothesis, a double *dcl* mutant (*Δdcl-1/2*) was prepared and infected by NcFV1. Consequently, the double mutant (*Δdcl-1/2*) showed much greater susceptibility to NcFV1 than the single *dcl* mutants or wild type, indicating the redundant function of the two Dicers against this virus (Fig. 4a). To examine similar redundancy in Argonaute and RDR, we created their double- (*Δqde-2/Δsms-2*) and triple (*Δqde-2/ΔSad-1/Δrpp-3*) deletion mutants, respectively. No or slightly elevated (~1.9-fold) accumulation of NcFV1 dsRNA and ssRNA was observed in the double Argonaute mutant (Fig. 4a, *Δqde-2* and *Δsms-2*) compared to the *qde-2* single mutant (*Δqde-2*) showing that QDE-2, but not SMS-2, predominantly functions in the antivirus RNAi against this virus. Similarly, NcFV1 accumulated in a triple *rdr* mutant (*Δqde-1/ΔSad-1/Δrpp-3*) at a level similar to that in their single-deletion mutants, suggesting no involvement of these *rdr* genes in the antiviral RNAi.

By contrast, *Δrpp-3, Δdcl-2*, and *Δqde-2* showed only ~1.4-fold or <3.7-fold increased accumulation levels of NcPV1 genomic dsRNA or its transcripts compared with any other single-deletion mutants (Fig. 4b and Supplementary Figure 3b). No significant additional increase in NcPV1 accumulation, relative to *Δrpp-3, Δdcl-2, and Δqde-2*, was observed in the Dicer double and RDR triple mutants. The Argonaute double mutant *Δqde-2/Δsms-2*, manifested two-to-threefold increased transcript levels relative to the single mutants (Fig. 4b). Similarly, no over twofold change in RnPV2 genomic dsRNA accumulation in the tested mutants from that in the wild-type strain was observed (Fig. 4c and Supplementary Figure 3c). When compared with the wild-type strain, no >1.7-fold increased accumulation of RnPV2 transcripts was detected, whereas a two-to-threefold decrease in RnPV2 transcript level was observed in *Δqde-2, Δdcl-1/2*, and *Δqde-2/Δsms-2* (Fig. 4c), a phenomenon warranting further investigation.

Collectively, we demonstrated that two Dicers DCL-1 and DCL-2 and one Argonaute QDE-2 play a major role in antiviral RNAi against at least a fusivirus in the vegetative phase of *N. crassa*.

Transcriptional and post-transcriptional regulation of host genes upon virus infection. Many genes including those of the RNAi pathway and putative antiviral response were previously...
reportedly to be induced at the transcription level by transgenic dsRNA expression in *N. crassa*. In another filamentous ascomycetous fungus, *C. parasitica*, infection by some RNA viruses was shown to induce similar homologous genes. Thus, it was expected that virus infection triggers transcriptional changes in the aforementioned virus hunting (see Supplementary Table 1).

To elucidate the transcriptional activation of RNAi-related genes upon virus infection, we performed chromatin immunoprecipitation (ChIP) followed by RT-qPCR. Dimethylation of histone H3 Lys 4 (H3K4me2) and Ser 5 phosphorylation of the RNA polymerase II C-terminal domain (Pol II S5P-CTD) are well-characterized as transcriptionally activated markers. As expected, significant and moderate accumulation of H3K4me2 and Pol II S5P-CTD was observed at the *dcl-2* and *qde-2* loci, respectively (Fig. 5c). However, the *rrp-3* gene locus and the noninducible genes *dcl-1* and *qde-1* loci did not appear to be enriched by H3K4me2 and Pol II S5P-CTD, when NcFV1 or NcPV1 was present (Fig. 5c). RnPV2 led to modest accumulation of Pol II S5P-CTD, but not H3K4me2, in the *rrp-3* gene locus and the noninducible gene *dcl-1* locus (Fig. 5c). Alternative transcriptional activation or post-transcriptional RNA processing might be involved in the increase of the *rrp-3* mRNA.

The results prompted us to examine their protein accumulation levels upon virus infection. We created strains expressing FLAG-octapeptide-tagged DCL-2, QDE-2, or RRP-3 from their endogenous loci and infected them by NcFV1, NcPV1, and RnPV2. Western blotting revealed that DCL-2 expression was strikingly elevated upon virus infection (Fig. 5d). Surprisingly, QDE-2 was shown to accumulate much less in the strain infected by NcFV1, but not in that by NcPV1 or RnPV2, than in the virus-free standard strain (Fig. 5d), suggesting NcFV1-specific post-transcriptional downregulation of qde-2. This downregulation may result from a counterdefense response targeting QDE-2 directly or indirectly by NcFV1. Taken together, these combined results clearly show transcriptional and post-transcriptional regulation of key genes of the antiviral RNAi in *N. crassa* upon virus infection.
Fusariviruses, a group of potential capsidless (+)ssRNA viruses that are distantly related to hypoviruses (family Hypoviridae) and proposed as the family "Fusariviridae", were detected in different Neurospora spp. of different geographical origins such as the United States (Louisiana), Haiti, and Indonesia, suggesting its widespread nature in members of the genus Neurospora (Fig. 2). Only a few fusariviruses that have been molecularly and biologically investigated, among which are Fusarium graminearum virus 1 (FgV1) strain DK2135-37 and Rosellinia necatrix fusarivirus 1 strain NW10 (RnFV1)58. These two fusariviruses appear to differ in genome organization and gene expression strategy. FgV1 has four open-reading frames (ORFs) and the three downstream ORFs are expressed via subgenomic RNAs, while RnFV1 has four open-reading frames (ORFs) and the three downstream ORFs are expressed via subgenomic RNAs, while RnFV1 resembles RnFV1 in terms of the 2-ORF genome organization. Another difference from FgV1 was detected in host factor requirement. A Fusarium graminearum (family Nectriaceae) gene product, hexagonal peroxisome (Hex1) protein, was identified as...
necessary for efficient FgV1 replication and normal symptom induction. Hex1 is a major component of the Woronin body, a peroxisome-derived organelle that feeds the septal pore under hyphal wounding stress and prevents the extension of wound-induced damage to neighboring cells. In this study, we examined the possible effects of deletion of the orthologous hex-1 gene of *N. crassa* on NcFV1 replication. However, no discernable effect was observed (Supplementary Fig. 3), suggesting that the involvement of HEX1 in the fusarivirus replication is specific to the pathosystem: *F. graminearum*/FgV1. *N. crassa* is the first organism to be used for genetic dissection of the RNAi pathway in fungi. There are two types of RNAi: quelling and MSUD (meiotic silencing by unpaired DNA). Quelling corresponds to cytoplasmic mitotic transgene RNAi in other eukaryotes. *N. crassa* has two Dicer (dcl-1 and dcl-2), three RDR (qde-1, Sad-1, and rrp-3), and two Argonaute genes (qde-2, smr-2), and two Dicer in antiviral RNAi, and no redundancy was found in Dicer in antiviral RNAi, and *FgAGO1* is homologous to *rrp-3* and its ortholog *rrp-3* of *N. crassa* are dispensable for antiviral RNAi, even though *dcl-2* and *agl-2* function in antiviral RNAi. Further studies using the *N. crassa* mutant will be of interest to explore the mechanism of NcFV1-mediated post-transcriptional regulation.

FgV1 is a ssRNA virus with two Dicers (FgDicer1 and FgDicer2) and two Argonautes (FgAGO1 and FgAGO2) that mediate RNAi in *N. crassa* and from antiviral RNAi in a model host filamentous fungus, *C. parasitica*. Similarities to two other ascomycetes, *Sclerotinia sclerotiorum* (family Sclerotiniaceae) and *Fusarium graminearum*, suggest that the dicing activity of Dicers is not required for the induction of RNAi. In contrast, an interesting difference was observed in the degree of induction: dcl-2 of *C. parasitica* was induced more highly than that in *N. crassa*, i.e., ~40-fold vs. ~8-fold, whereas the induction of rrp-3 and its ortholog dcl-2 in the two fungi, i.e., 20–30-fold. However, *C. parasitica* DCR4 seems to be not fully functional due to a nonsense mutation in any of the three alternatively splicing variants of transcripts. Thus, the biological significance of high transcriptional induction of dcr4 in *C. parasitica or rrp-3* in *N. crassa* remains elusive. In *C. parasitica*, no redundancy was found in Dicer in antiviral RNAi, and *dcl2* and *agl2* transcript levels were increased 10–40-fold upon virus infection. By contrast, two Dicer genes, *dcl-1* and *dcl-2*, in *N. crassa* played redundant roles (Fig. 4a), and their transcript levels were augmented by less than 7-fold (Fig. 5b and Supplementary Fig. 4). The high transcriptional upregulation of *dcl-2* might have been compromised by the redundancy of Dicer during the course of evolution of filamentous fungi.

Different patterns between the accumulation of the partitiviruses (NcPV1 and RnPV2) and NcFV1 in an array of mutant *N. crassa* strains were observed. In the double *dcl* mutant or *qde-2* mutants, NcFV1 dsRNA-replicative form accumulated approximately 10-fold relative to the wild-type strain, and this increase was more pronounced when NcFV1 ssRNA was compared (Fig. 4, Supplementary Fig. 3). Such an elevation in the two mutants was not observed for NcPV1 or RnPV2 dsRNA accumulation (Fig. 4, Supplementary Fig. 3). It was previously shown that certain partitiviruses are tolerant to antiviral RNAi, despite their ability to induce RNAi, and accumulate at a similar level in RNAi-competent and -deficient *C. parasitica* strains. A similar phenomenon was also observed for a capsidless (+)ssRNA hypovirus. Thus, the failure of NcPV1 and RnPV2 to accumulate more in *dcl-1/2* and *qde-2* mutants than in the wild-type strain is not surprising and suggests their evolution of ways to evade antiviral RNAi.

Collectively, this study has opened up an avenue in modern virology, and shall accelerate its advance with available molecular tools and biological resources. This has great impact on studies with viruses of other fungi, particularly plant pathogenic ascomycetes that share many homologous genes with *N. crassa*. Some plant fungal diseases such as chestnut blight are targets of biological control using viruses infecting the pathogenic fungi so-called "virocontrol". Filamentous fungi have multilayered antiviral defense impairs virocontrol: RNAi working at the cellular level and vegetative incompatibility functioning at the population level. Better understanding of antiviral defense vs. viral counterdefense and fine-tuning of expression of associated genes are prerequisite for their successful virocontrol. In this regard, further studies using the *N. crassa*/viruses, e.g., aiming at exploring antivegetative incomparability responses evoked by viruses should contribute to virocontrol of phytopathogenic fungi.

We discovered post-transcriptional downregulation of an Argonaute QDE-2 specifically upon infection by a (+)ssRNA virus (NcFV1) in *N. crassa* (Fig. 6). A plant (+)ssRNA virus is known to encode an RNAi suppressor that induces an autophagy pathway targeting an Argonaute (AGO1). It will be of interest to explore the mechanism of NcFV1-mediated post-transcriptional downregulation of *qde-2* (Fig. 5d). It is anticipated that NcFV1 encodes an RNAi suppressor whose mode of action is different from those of the suppressors from the prototype hypovirus (Cryptonectria hypovirus 1 p29) and a fusarivirus (FgV1 ORF2 protein) that transcriptionally downregulate RNAi key genes. Other interesting future challenges include to investigate whether meiotic silencing serves as antiviral defense, how the virus or dsRNA is sensed and triggers antiviral RNAi, how the virus impedes host fungal vegetative incompatibility, and what host factors are associated with viral replication and symptom induction.

### Methods

**Fungal and viral materials.** The fungal strains tested in this study are summarized in Table 1 and Supplementary Table 2. Many *N. crassa* strains were purchased from the Fungal Genetics Stock Center (FGSC) (http://www.fgsc.net) and Neurospora protocols available in the website were used unless otherwise mentioned. Full names and accession numbers of mycoviruses detected in this and previous studies are provided in Table 1. *R. neoartica* strains W57 (infected by RnPV2) and *C. parasitica* strain 9821 (infected by MyRv1) were described earlier. These fungal strains were grown on Difco PDA plates for maintenance unless otherwise mentioned.
Neurospora knock-in (KI) and KO strains (Supplementary Table 2) were prepared by the standard method as described earlier, with specific primers summarized in Supplementary Table 3.

Experimental virus introduction into N. crassa. Virion transfection was performed as described by Hillman et al. First, virus particles ofRpc2V and MyrV1 were prepared as described by Chiba et al. and Hillman et al. Protoplasts of the N. crassa standard strain (74-OR23-1VA, FGSC #2489) were prepared by the method of Eusebio-Cope and Suzuki as it is generally applicable to protoplast preparation of ascomycetous fungi. Briefly, liquid cultures of N. crassa were harvested and incubated in a cell-wall digestion solution containing β-glucoronidase and lyzing enzyme (Sigma-Aldrich). Protoplasts (0.5 × 10^7 cells in 200 µl) were mixed with purified virus particles in the presence of polyethylene glycol (PEG) and CaCl_2 ion. Protoplast fusion was performed between N. intermedia (FGSC #259) and N. crassa as described by Shahi et al. For this purpose, the N. crassa standard strain (74-OR23-1VA) was transformed by the NTC (nourseothricin)-resistance gene and used for subsequent screening. An equal number of protoplasts (~2 × 10^9) from the two fungal strains were fused with the aid of PEG/CaCl_2. The protoplast fusants were grown regenerated on the regeneration media for 1 day and subsequently on overlaid top agar containing 30 µg/ml NTC for 2–3 days for screening the N. crassa recipient. Protoplast regenerants resistant to NTC were transferred into PDA plates containing 30 µg/ml NTC and incubated for 1 day before detecting NiFV1. After confirming the presence of NiFV1 by the one-step colony PCR method, NiFV1-positive colonies were anastomosed with the original nontransformed N. crassa strain. A mycelial plug taken from the recipient side was again anastomosed with the original recipient. This hyphal fusion step was repeated three times.

The virus-infected N. crassa helper-5 strain (FGSC #8747; amat his-3 pk1 hph cyh-1, Bmil pan-2) was created as a donor strain for virus horizontal transfer to a series of recipient strains. Specifically, the virus-free helper-5 strain and the virus-infected N. crassa wild strain (FGSC #10950, NcVE1; FGSC #3975, NcPV1) were cocultured into a slant of Vogel’s sucrose medium containing 10 µg/ml pantothenic acid and 25 µg/ml histidine. Subsequently, the virus-infected heterokaryon was forced by a passage into a slant of minimal medium containing 1.5 µg/ml benomyl and then the virus-infected helper-5 strain was purified through microcentrifugation preparation on SC medium containing 1 mM iodacetate. Similarly, the standard wild-type strain (74-OR23-1VA) and the derived KO and KI strains were infected by forcing heterokaryon with the virus-infected helper-5 strain and then were extracted by spreading onto a plate of Vogel’s FGS medium containing 5 µM FUDR (5-fluorodeoxyuridine) and 1 mM uracil after microcentrifugation preparation. A schematic diagram for the manipulation was described in Supplementary Fig. 2.

RNA analyses. The total RNA and dsRNA fractions were modified by the method of Eusebio-Cope and Suzuki. Specifically, Neurospora strains were grown with shaking in Vogel’s minimal medium N at 32 °C for 2 days and were harvested by filtration. Tissues were transferred into 22-ml screw-capped tubes (Sarstedt, 72.694) containing 0.40.6-mm zirconia beads, 450 µl of 2× STE, 50 µl of 10% SDS, and 300 µl of phenol/chloroform, and were extracted by using the Minilys homogenizer (Bertin Instruments) for 30 s at maximum speed. After centrifugation at 15,000 rpm for 5 min at 4 °C, 500 µl of the upper phase was transferred into a 1.5-ml microtube and 50 µl of 3 M sodium acetate and 400 µl of isopropanol were added. The concentrations of total RNA were measured by using the Qubit RNA Assay Kit (Thermo Fisher), and the qualities were confirmed by agarose gel electrophoresis. For dsRNA purification, equal amounts of total RNA were dissolved in 420 µl of 1× STE after isopropanol precipitation, then incubated in a 65 °C water bath for 15 min, and quickly chilled on ice. Then, 80 µl of ethanol were added as described by Okada et al. to purify dsRNA. Complementary DNA (cDNA) was prepared by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo), and quantitative PCR was performed three times using THUNDERBIRD Probe qPCR Mix (Toyobo) and a LightCycler 96 system (Roche Diagnostics) with specific primers for the virus-free standard strain as 1. Dioxigenin-labeled DNA probes for N. crassa genes were prepared by genomic PCR and used in northern blotting according to the manufacturer’s instructions. See Supplementary Table 3 for the primer sequences.
In silico detection of viral sequences. RNA-seq data were obtained from NCBI. After quality trimming by trimmomatic, the reads were mapped to genomic DNA, gDNA, mtDNA, and rRNA of the standard N. crassa strain 74-OR23-1VA with Bowtie2 to obtain unmapped reads. Unmapped reads were then assembled de novo with Trans-AbYSs (https://www.bcgsc.ca/resources/software/trans-abys) into contigs. To create local fungal virus database, potential fungus was extracted from current virus database (NCBI: taxid10239). The candidates of fungal viral sequences were selected from the contigs by a BlastX search (E value < 1 × 10⁻³) using the local fungal virus database.

High-throughput sequence analysis of a N. intermediate strain. The dRNA-enriched fractions were obtained from petri dish-grown mycelia of a strain of N. intermediate (FGSC # 2559, H2125-1) as described by Chiba et al. The dRNA preparation (17.8 ng/µl) was subjected for dDNA library construction using the TrueSeq Stranded Total RNA LT Sample Prep (Illumina, San Diego, CA, USA) and then next-generation sequencing in Illumina technology (HiSeq 2000, 100-bp paired-end reads). Raw data for this project were deposited in NCBI Sequence Read Archive (SRA) under accession No. DRR248874. The cDNA library construction and deep-sequencing analysis were performed by Macrogen Japan, Ltd. A total of 56,528,326 paired-end reads (5,709-Mb read sequences) were assembled into 8415 contigs (~7760 nt in length, average 983 nt) using de novo assembler of CLC Genomics Workbench (version 11; CLC Bio-Qiagen). These contigs were subsequently used as queries for a local BLAST search against the RefSeq annotated viral genome database.

Chromatin immunoprecipitation (ChIP) assay and western blotting. We followed the procedures of the ChIP assay and Western blotting as described previously. The following antibodies were used: anti-H3K4me2 antibody (active motif, 39141), anti-RNA polymerase II CTD repeat YSPTSPS (phospho 5S) antibody (Abcam, ab5131), anti-alpha-tubulin antibody (Sigma-Aldrich, T6199), and anti-FLAG antibody (MBL, M185-3). RT-qPCR experiments were performed two times using FAST SYBR Green master kit (KAPA) with the listed primers (Supplementary Table 3) and analyzed using a LightCycler® 96 System (Roche Diagnostics). The standard N. crassa strain (74-OR23-1VA) was engineered such that rrp-3-Flag, dci-2-Flag, or gde-2-Flag was knocked in. Alpha-tubulin detected by Western blotting with anti-alpha-tubulin antibody was used as a loading control.

Differential gene expression (DEG) analysis. DEG analysis was performed by the standard HISAT-StringTie-Ballgown pipeline. Based on the results of in silico detection of virus-candidate sequences, we choose ten virus-infected strains (SRR089835, SRR098940, SRR797998, SRR798015, SRR798021, SRR798029, SRR798030, SRR798051, SRR798054, and SRR798057) and ten highly likely virus-free strains (SRR797950, SRR797951, SRR797953, SRR797955, SRR797956, SRR797961, SRR797962, SRR797964, SRR797965, and SRR797967) and compared their levels of gene expression.

Phylogenetic analyses. The sequences of mycovirus used in this study were obtained from the NCBI website and are summarized in Supplementary Table 4. For the RdRP or CP sequences,we selected from the contigs by a BlastX search from current virus database (NCBI: taxid10239). The candidates of fungal viral sequences were selected from the contigs by a BlastX search (E value < 1 × 10⁻³) using the local fungal virus database.

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Author contributions
N.S. initiated the project. N.S. and S.H. conceived the study and designed the experiments. S.H. introduced viruses into mutant fungal strains, and comparatively analyzed them by western blotting, ChIP, and RT-qPCR. A.Y. performed RT-qPCR and dsRNA extraction. A.E.C. and S.S. designed and performed virion transfection and gel electrophoresis. S.H. and S.M. mined public databases for viral sequences and phylogenetic analysis. They also compared the viral genome sequences by RACE and northern blotting and dsRNA gel electrophoresis. S.H. and S.M. performed computational analysis of the entire virus genome sequences using the Bowtie2 program on functional genomics. They also developed MapTest-Ng for the analysis of DNA and protein evolutionary models. N.S. and S.H. wrote and reviewed the paper.

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Competing interests
The authors declare no competing interests.

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