Supplementary Information for

Argonaute-independent, Dicer-dependent antiviral defense against RNA viruses

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SI Materials and Methods

Search for Argonaute domains

AGL1, AGL2, AGL3, and AGL4 (GenBank accessions: ACY36939.1, ACY36940.1, ACY36941.1, and ACY36942.1, respectively) are encoded by *agl1*, *agl2*, *agl3*, and *agl4*, respectively. The database Pfam 35.0 database (1) was searched for the conserved domain with these AGL amino acid sequences by hmmscan in HMMER version 3.3.2 (e-value < 0.001) (https://www.mankier.com/package/hmmer) (2). The domain positions were mapped by using ggplot2 version 3.4.2 (https://ggplot2.tidyverse.org/).

Vector construction

All vectors were constructed with In-Fusion HD Cloning kit (TaKaRa Bio). The polymerase chain reaction (PCR) insert was prepared with primers and template listed in *SI Appendix*, Table S1 by KOD -Plus- Neo (TOYOBO) (or PrimerSTAR GXL DNA polymerase [TaKaRa Bio] for one construct; see below) according to the manufacturer's instructions. The vectors were linearized with restriction enzymes provided by New England Biolabs, TOYOBO, or TaKaRa Bio, according to the manufacturer's instructions.

For fungal gene disruption, three backbone vectors for homologous recombination (HR) were generated—pGEM::loxPneoR, pGEM::loxPhygRinv, and pGEM::loxPntcRinv—which contain the antibiotic resistance gene as a selectable marker gene (SMG) cassette against neomycin, hygromycin, or nourseothricin (NTC), respectively, and a *loxP* sequence at both ends. For pGEM::loxPneoR, the neomycin resistance gene cassette (*neoR*) (3) was amplified from pCPXNeo (4), with primers that contain the *loxP* sequence. The amplified *neoR* was cloned into the *Eco*RI sites of the pGEM-T Easy vector (Promega). For pGEM::loxPhygRinv, the hygromycin-resistance gene cassette (*hygR*) was amplified from pCPXHY3 (5). The *hygR* amplicon was replaced by *neoR* in the pGEM::loxPneoR linearized at the two *Eco*RI sites and placed between *neoR* and *loxP* at both ends. Because the *loxP* sequence is repeated, the cloning reaction can produce two types of products, with *hygR* in the same or inverse orientation compared to the orientation of *neoR* in pGEM::loxPneoR. The latter one (inverse orientation) was used; thus, "inv" was added to the vector name. For pGEM::loxPntcRinv, the NTC resistance gene cassette (*ntcR*) was amplified from pAL12-Lifeact (6). Then, the *ntcR* amplicon was then was replaced for the promoter and coding sequence of *hygR* in pGEM::loxPhygRinv linearized with *Eco*RI and *Bam*HI.

To construct vectors for disruption of the target genes, approximately 0.75 kilobase pair (kbp) upstream and downstream flanking regions (5′ and 3′ arms, respectively) of the target genes were respectively cloned into the *Sph*I and *Sal*I sites of the HR backbone vectors. The homology arm sequences were obtained from the *Cryphonectria parasitica* EP155 v2.0 database (https://mycocosm.jgi.doe.gov/Crypa2) provided by the Joint Genome Institute (JGI). The homology arms for *dcl1* and *dcl2* were cloned into pGEM::loxPhygRinv. The homology arms for *agl1*, *agl2*, *agl3*,

and *agl4* were cloned into pGEM::loxPneoR, pGEM::loxPhygRinv, and/or pGEM::loxPntcRinv for multiple gene disruption (*SI Appendix*, Figs. S1 and S2*A*). For cloning into the pGEM::loxPneoR vector that contains the *Sph*I site in *neoR*, the 5′ and 3′ arms were first sub-cloned into the *Sph*I and *Sal*I sites of the pGEM-T Easy backbone without *neoR*. Then, the sub-cloning vectors were linearized with *Eco*RI and connected with the *neoR* (P_{gpd}-*nptII*-T_{tub}) amplicon.

The plasmid vector termed pCPXHY3::Cre was generated to introduce Cre recombinase into *C. parasitica*. The Cre coding sequence (CDS) was amplified from pKAES175 (7) and cloned into the *Not*I and *Sph*I sites of pCPXHY3.

The plasmid vector termed pNeo3::P_{dcl2}-*dcl2* was generated for genetic complementation of *dcl2*. Prior to cloning, the backbone vectors pXYLHY3 and pXYLNeo3 were generated for use in other studies. pXYLHY3 has a xylose-responsive promoter (P_{xyl}) from *Colletotrichum orbiculare*, while pCPXHY3 has a constitutive expression promoter (P_{gpd}) from *C. parasitica*. The promoter P_{xyl} was amplified from pCB1636_lox_HPT-TK (8) using a primer with the *Spe*I and *Xho*I sequences to enable replacement of P_{xyl} with any other promoter. Then, the P_{xyl} amplicon was then cloned into the *Xcm*I and *Hind*III sites of pCPXHY3 to replace P_{gpd}. pXYLNeo3 was generated by replacing *hygR* in pXYLHY3 with *neoR*. For the replacement, *neoR* was amplified from the pCRPNeo vector. Then, the *neoR* amplicon was cloned into pXYLHY3 linearized with *Eco*RI and *Xba*I to replace *hygR*. The vector pNeo3::P_{dcl2}-*dcl2* was generated by replacing P_{xyl} in pXYLNeo3 with the *dcl2* gene with its native promoter (2000 bp upstream of the start codon, the region transcriptionally responsive to viral infection (9)) (P_{dcl2}-*dcl2*). For the replacement, P_{dcl2}-*dcl2* was amplified from the genomic DNA of *C. parasitica* EP155 by PrimerSTAR GXL DNA polymerase (TaKaRa Bio) and cloned into the *Xho*I and *Sph*I sites of pXYLNeo3.

Fungal transformation

Spheroplasts of *C. parasitica* were prepared as described previously (10), with minor modifications to the original protocol (11). For gene disruption, each HR cassette was amplified from the plasmid vector described above by using the forward primer for the 5′ arm and the reverse primer for the 3′ arm (*SI Appendix*, Table S1) by KOD -Plus- Neo (TOYOBO). The amplicon was purified with Wizard SV Gel and PCR Clean-Up System (Promega). The purified amplicon (5 μg) was introduced into the fungal spheroplasts (1–2 × 10^7 cells/mL × 100 μL) by a method mediated by a polyethylene glycol (PEG)-mediated method, as described previously (11). Single colonies harboring *neoR*, *hygR*, or *ntcR* were selected with 20 μg/mL G418 sulfate, 40 μg/mL hygromycin B, or 20 μg/mL NTC, respectively, in 1% top agar layered on regeneration medium containing regenerated spheroplasts. The single colonies on the top agar were further selected on PDA containing each antibiotic at the same final concentration. Among the antibiotic-resistant colonies on PDA, the deletion mutants were screened by direct colony PCR with Quick Taq HS DyeMix (TOYOBO). The screening was performed with combinations of a forward primer targeting the upstream region outside the 5′ arm on the fungal genome

and a reverse primer targeting the SMG. The screened mutants were further cultured on PDA with antibiotic under sunlight to induce conidiation. Single conidia (monokaryotic) were isolated on new antibiotic PDA to remove wild-type karyotypes. The complete deletion of the target genes was further validated by PCR and Southern blotting as described below.

For single to triple gene disruption, three different SMGs (*neoR*, *hygR*, and *ntcR*) were utilized, as summarized in *SI Appendix*, Fig. S2*A*. To generate Δ*dcl1* and Δ*dcl2*, *dcl1* and *dcl2* were replaced with *hygR* in the DK80 background. To generate Δ*agl1* to Δ*agl4*, *agl1* to *agl4* were replaced with *neoR* in the DK80 background. To generate the double mutant Δ*dcl2*Δ*agl2*, *agl2* was replaced with *neoR* in the Δ*dcl2* background. To generate the triple mutant Δ*agl1/3/4*, *agl4* was first replaced with *hygR* in the Δ*agl1* background, and then *agl3* was replaced with *ntcR*.

For quadruple and quintuple gene disruption, the SMGs were removed by the Cre-*loxP* system (12). First, the *C. parasitica* transformants that produce functional Cre recombinase were generated (*SI Appendix*, Figs. S2*B* and S2*C*). Δ*agl4* (*agl4* disruptant with *neoR*) was transformed with pCPXHY3::Cre by the PEG-mediated method described above (for plasmid, 10 μg was used for each transformation instead of 5 μg PCR amplicon). This transformant is termed Δ*agl4*+*Cre*. The transformants were selected based on hygromycin resistance, genotyping, and Cre activity. The Cre activity was estimated by PCR targeting an upstream region of the 5′ arm to the end of the 3′ arm of *agl4* (*SI Appendix*, Fig. S2*B* and Table S2). The transformant showing complete excision of *neoR* (Δ*agl4*+*Cre* #10) (*SI Appendix*, Fig. S2*C*) was selected as a Cre donor for the following anastomosis-mediated SMG excision (12) (*SI Appendix*, Fig. S2*D*). Briefly, the Cre donor (hygromycin resistant) was cultured alongside Δ*agl1/3/4*, which is resistant to all three antibiotics (neomycin, hygromycin, and NTC) for horizontal transfer of Cre recombinase via hyphal anastomosis. After co-culturing for 2 weeks, a small (1 mm^3) mycelial plug was taken from the Cre recipient side adjacent to the contact zone at the colony periphery. The mycelial plug was transferred to PDA without antibiotics and grown under sunlight to induce conidiation. The generated conidia were suspended in sterile water and spread on fresh antibiotic-free PDA for isolation. The single conidial sub-isolates were transferred to PDA supplemented with 50 μg/mL hygromycin to screen for the gene disruptants that lost *hygR* and to exclude the hygromycin-resistant Cre donor that is resistant to hygromycin. The screening yielded a Δ*agl1/3/4* strain that lost *hygR* but kept *neoR* and *ntcR*. This Δ*agl1/3/4* strain was used for the analyses in this study. Then, Δ*aglQ* was generated by replacing *agl2* with *hygR* in this Δ*agl1/3/4* background. The SMGs in Δ*aglQ* were removed by the same Cre-*loxP* system (*SI Appendix*, Fig. S2*D*). The Δ*aglQ* strain, which lost *hygR* and *neoR* but kept *ntcR* was obtained and used for the analyses in this study. For the SMG removal experiments, approximately 100–500 independent conidial sub-isolates were screened. Then, Δ*dcl2*Δ*aglQ* was generated by replacing *dcl2* with *hygR* in the Δ*aglQ* background.

The *dcl2* complementation strain was generated in Δ*dcl2*Δ*aglQ*, namely Δ*dcl2*Δ*aglQ*+*dcl2*, by transforming Δ*dcl2*Δ*aglQ* with pNeo3::P_{dcl2}-*dcl2* by the PEG-mediated method as described above. The transformants were selected for the neomycin resistance, genotype, and the DK80-like tolerance to

RnVV1.

Virus inoculation

Virus-free strains were inoculated with viruses mainly via hyphal fusion/anastomosis (Fig. 2*A*) (13). A small mycelial plug (≈1 mm^3) from each virus donor fungal strain and each virus-free recipient fungal strain (Table 1 and *SI Appendix*, Table S3) was placed onto a central part on PDA (9 cm in diameter) with a distance of around 5 mm^3 between the donor and the recipient. The donor and recipient were cultured for approximately 1 week to allow hyphal anastomosis between the two fungal strains and consequent horizontal virus transfer to the recipient side. After the dual culture, a small mycelial plug (≈1 mm^3) was excised from the recipient side at the farthest part from the donor and recipient hyphal contact. The excised mycelial plugs were transferred to new PDA and sub-cultured for a few days for further analyses. For CHV1 and CHV1-Δp69, which severely inhibit recipient hyphal elongation and may induce contamination by donor hyphae, recipient colonies were repeatedly fused with the virus-free recipients.

For one experiment (Fig. 2*E*), RnVV1 was inoculated by virion transfection (Fig. 2*D*) (14). Crude virus particles of RnVV1 were extracted from *C. parasitica* EP155Δ*dcl2*/RnVV1 (15), as described previously (16), by omitting the sucrose gradient fractionation and the following procedures. The crude virus particles were purified by filtration through a 0.22 μm pore size filter (Millipore) by centrifugation. The flow-throw was used to transfect spheroplasts of DK80, Δ*dcl2*, and Δ*agl2*, as described previously (10), by omitting the hygromycin selection step. Virus-infected colonies were screened by direct colony reverse transcription–polymerase chain reaction (RT-PCR) (17) with the Prime Script One Step RT-PCR Kit, Ver.2 (Dye Plus), as described previously (18).

PCR genotyping and Southern blotting

Genomic DNA was extracted from fungal mycelia cultured on PDA-cellophane for 2 or 3 days. Briefly, the mycelia were ground with a mortar and pestle in liquid nitrogen. The frozen mycelial powder was mixed with a 10-fold volume (per fresh mycelial weight) of a nucleic acid extraction buffer (50 mM NaCl, 100 mM Tris-HCl [pH 8.0], 10 mM ethylenediaminetetraacetic acid [EDTA, pH 8.0], and 0.5% sodium dodecyl sulfate [SDS]). Total nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (PCIA, prepared with phenol saturated with TE buffer [pH 7.9]) followed by chloroform/isoamyl alcohol (CIA) extraction. The crude total nucleic acids were treated with 10 μg/mL RNase A (Sigma-Aldrich Co., LLC) at 37°C for 30 min, purified by PCIA and CIA extraction followed by 2-propanol precipitation and rinsing with 70% (v/v) ethanol, and resuspended in TE buffer. The concentration of the purified DNA was measured with a NanoDrop spectrophotometer.

PCR genotyping was carried out in 10 μL of Quick Taq HS DyeMix (Toyobo, Co. Ltd.) with 10 ng of purified genomic DNA and primers listed in *SI Appendix*, Table S2 according to the manufacturer's instructions (three-step cycle). PCR products were electrophoresed in 1.0% (w/v) agarose gel in 0.5×

TAE and stained with ethidium bromide (EtBr). For all gel electrophoresis, the size of the PCR products was checked with GeneRuler 1 kb DNA Ladder (Thermo Scientific).

For Southern blotting, 8–10 μg (constant amount for each gel) of genomic DNA was digested with restriction enzymes (New England Biolabs or TOYOBO) overnight at the temperature recommended by the manufacturers. The *dcl1* and *dcl2* loci were analyzed with *Bam*HI; the *agl1*, *agl2*, and *agl4* loci with *Pst*I; and the *agl3* locus with *Xho*I (*SI Appendix*, Fig. S1). The digested DNA was purified by PCIA extraction followed by ethanol precipitation. All recovered purified DNA fragments were electrophoresed in a 1.0% (w/v) agarose gel with 0.5× TAE at constant 100 V. The electrophoresed DNA on the gel was blotted onto a nylon membrane Hybond-N+ (GE Healthcare) according to the manufacturer's instructions. Briefly, the gel was depurinated in 0.125 M HCl for 10 minutes with gentle agitation. The gel was then incubated in denaturation buffer (1.5 M NaCl and 0.5 N NaOH) for 30 min with gentle agitation, followed by incubation in neutralization buffer (1.5 M NaCl and 0.5 M Tris-HCl, pH 7.5) for 30 min with gentle agitation. Denatured DNA on the gel was transferred to Hybond-N+ by capillary blotting using filter papers immersed in 20× SSC (0.3 M trisodium citrate and 3 M NaCl). After overnight, the transferred DNA was crosslinked to the membrane by UVP Crosslinker CL-1000 at 240,000 μJ/cm^2. The target genomic region on the membrane was detected according to the DIG (digoxigenin) Application Manual provided by Roche Applied Science (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents/199/985/dig-application-manual-for-filter-hybridisation-iris.pdf). DIG-labeled DNA probes were synthesized with the PCR DIG Labeling Mix (Roche), the primers listed in *SI Appendix*, Table S1 (primer sets for *dcl1*-5′arm, *dcl2*-5′arm, *agl1*-5′arm, *agl2*-3′arm, *agl3*-5′arm, or *agl4*-5′arm for the respective loci), and plasmid templates containing the target genomic regions. Chemiluminescence was detected with the ImageQuant LAS 4000 (GE Healthcare).

RNA extraction, northern blotting, and RT-PCR

RNA was extracted from fungal mycelia cultured on PDA-cellophane for 3 days. Briefly, the frozen mycelial powder was mixed with a 10-fold volume (per mycelial fresh weight) of the nucleic acid extraction buffer described above and subjected to PCIA (prepared with phenol saturated with water) extraction, CIA extraction, and ethanol precipitation. The total nucleic acid pellets were rinsed with 70% ethanol, dried, and dissolved in sterile Milli-Q water. The total RNA fraction consisting of both dsRNA and ssRNA was purified by treating total nucleic acids with RQ1 RNase-Free DNase (Promega) at 37°C for 30 min followed by phenol-chloroform extraction, PCIA extraction, CIA extraction, ethanol precipitation, and rinsing with 70% ethanol, and then dissolved in sterile Milli-Q water. The RNA concentration was measured using the NanoDrop spectrophotometer. The total RNA fraction was used for northern blotting (Fig. 3*A*–*E*), RT-PCR (*SI Appendix*, Fig. S6), and small RNA-Seq. The ssRNA-enriched fraction was precipitated from the total nucleic acids with lithium chloride (LiCl, final concentration 2 M, which is supposed to enrich high molecular-weight ssRNA (19)), rinsed with 70%

ethanol, and dissolved in sterile Milli-Q water. The crude ssRNA-enriched fraction was used for northern blotting and RT-PCR (Figs. 2 and 3*F* and *G*, and *SI Appendix*, Figs. S5 and S7). The ssRNA-enriched fraction was further purified by treatment with RQ1 RNase-Free DNase followed by DNase purification as described above. The DNase-purified ssRNA-enriched fraction was used for RNA-Seq.

Northern blotting was performed with two different methods for the total RNA and ssRNA-enriched fractions. The total RNA fraction (5 μg per lane) was electrophoresed on a 1% agarose gel in 0.5× TAE and stained with EtBr after electrophoresis. The total RNA in the gel was denatured by alkaline treatment after electrophoresis and blotted onto a nylon membrane (Hybond-N+, GE Healthcare), as described previously (20). The ssRNA-enriched fraction (2 μg per lane for viral detection or 10 μg per lane for host mRNA and some RnVV1/MyRV2 detections; constant for each gel) was pre-stained with EtBr, denatured during electrophoresis in MOPS-formaldehyde gel, and blotted onto Hybond-N+ immediately after the electrophoresis, as described previously (21). Target RNA bands on the membranes were detected by using complementary DNA (cDNA) probes labeled with DIG by the PCR DIG Labeling Mix (Roche). Primers used for the cDNA probe preparation are listed in *SI Appendix*, Table S2. Plasmids (pGEM-T or pCPXHY3 backbone) containing cDNA of the target viral or host mRNA regions were used as templates.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with ReverTra Ace qPCR RT Kit (TOYOBO) and THUNDERBIRD SYBR qPCR Mix (TOYOBO) according to the manufacturer's instructions. Primers used for qRT-PCR are listed in *SI Appendix*, Table S2. The real-time PCR was performed in qTOWER3/G (Analytik Jena). The relative viral RNA level was calculated by the $2^{-\Delta\Delta CT}$ method with the host actin homolog (centractin ortholog) gene (*act2*) as internal control and DK80-infected with the respective viruses as a calibrator (22).

Semi-qRT-PCR was performed with Prime Script One Step RT-PCR Kit, Ver.2 (Dye Plus) (TaKaRa Bio) in a volume of 10 μL using 10 μg of purified total RNA or ssRNA-enriched fraction as a template with the primers listed in *SI Appendix*, Table S2. The standard three-step PCR was carried out according to the manufacturer's instructions. The PCR products were electrophoresed in 1.0% (w/v) agarose gel in 0.5× TAE and stained with EtBr. The relative band intensity was quantified with ImageJ 1.5.3t (https://imagej.nih.gov/ij/).

RNA-Seq and small RNA-Seq analyses

The RNA-Seq library was constructed with the KAPA mRNA Capture kit (Kapa Biosystems, #KK8440) and the MGIEasy RNA Directional Library Prep set (MGI, #1000006385) by GenomeRead Inc. The cDNA library was sequenced at a 17 million read depth of 2 × 150 bp paired-end by DNBSEQ-G400RS (MGI) by GenomeRead Inc. The RNA-Seq reads were subjected to quality control and adaptor trimming by fastp version 0.19.5 (23). The trimmed RNA-Seq reads were mapped to the masked genome sequence of *C. parasitica* strain EP155 (Cryphonectria_parasiticav2.nuclearAssembly.masked) (24) by HISAT2 version 2.2.1 (25). The output SAM files were converted into BAM files, sorted, and indexed by

SAMtools version 1.7 (26). The reads mapped to respective genes in a gene catalog (Cparasiticav2.GeneCatalog20091217.gff) (24) were counted by htseq-count version 2.0.2 with the options -f bam, -r pos, -i name, and -s reverse (27). Differentially expressed genes (DEGs) were analyzed by DESeq2 version 1.42.0 (28) with a minimum read threshold of one. Transcripts per million (TPM) were calculated according to the formula described previously (29). The DEG analysis results were visualized with ggplot2 version 3.4.3 (https://ggplot2.tidyverse.org/).

For small RNA-Seq analysis, total RNA extracted from virus-infected *C. parasitica* strains was sent to Macrogen Inc. The small RNA cDNA library was constructed using the NEBNext Small RNA Library Prep Set for Illumina (New England Biolabs) and subsequently sequenced in a single read (SE) (1 × 50 nt) run on the Illumina HiSeq 2500 platform. The adapter was trimmed from the raw reads (25~64 million reads); then, the reads were filtered for a size range of 16–32 nt using CLC Genomic Workbench (version 22; CLC Bio-Qiagen, Aarhus, Denmark). The retained reads in each library were then mapped to respective virus genome or genomic segments using the Read Mapping algorithm in the CLC Genomics Workbench. The virus-derived small RNA reads were used for further analysis with the MISIS-2 program (30).

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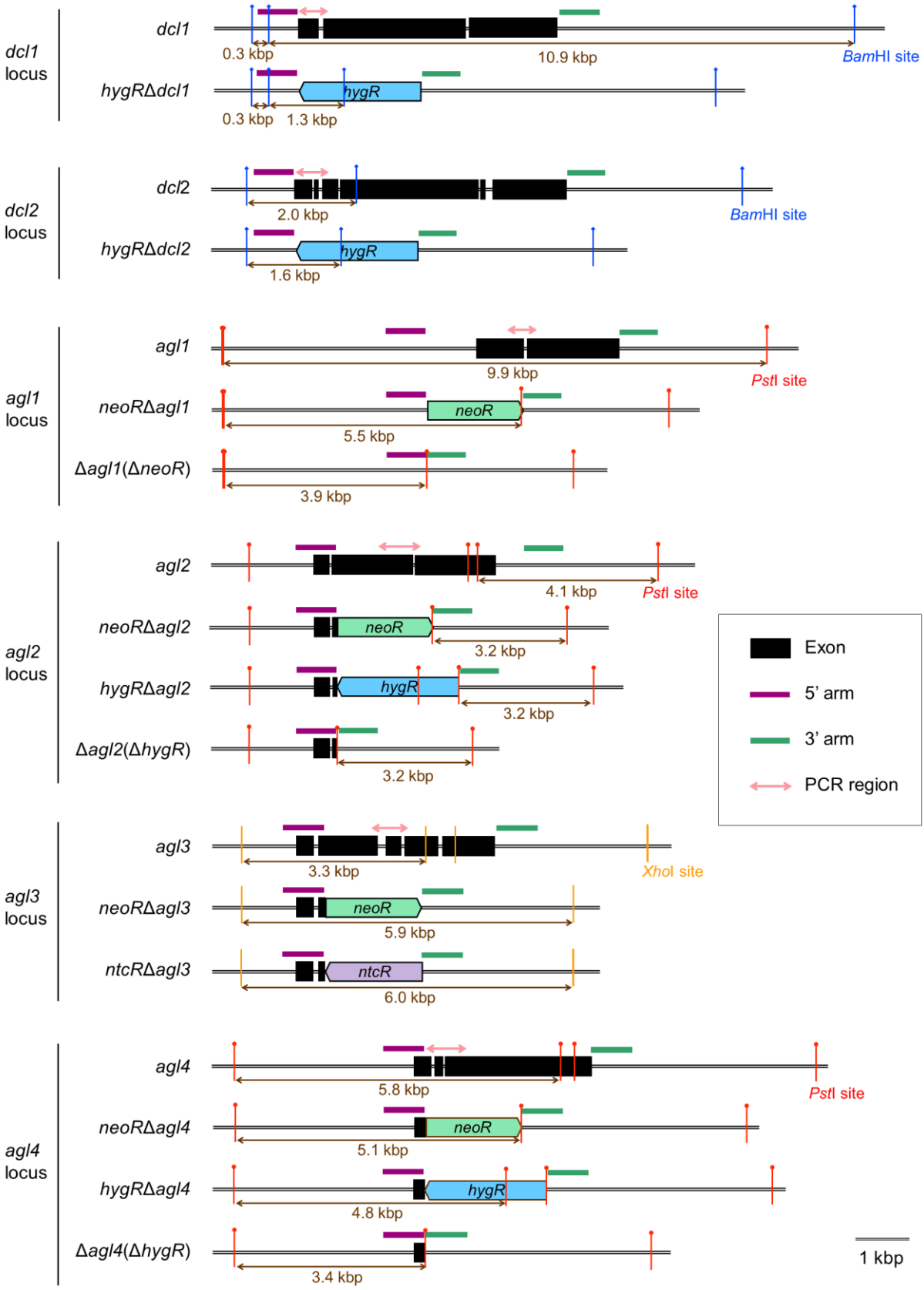
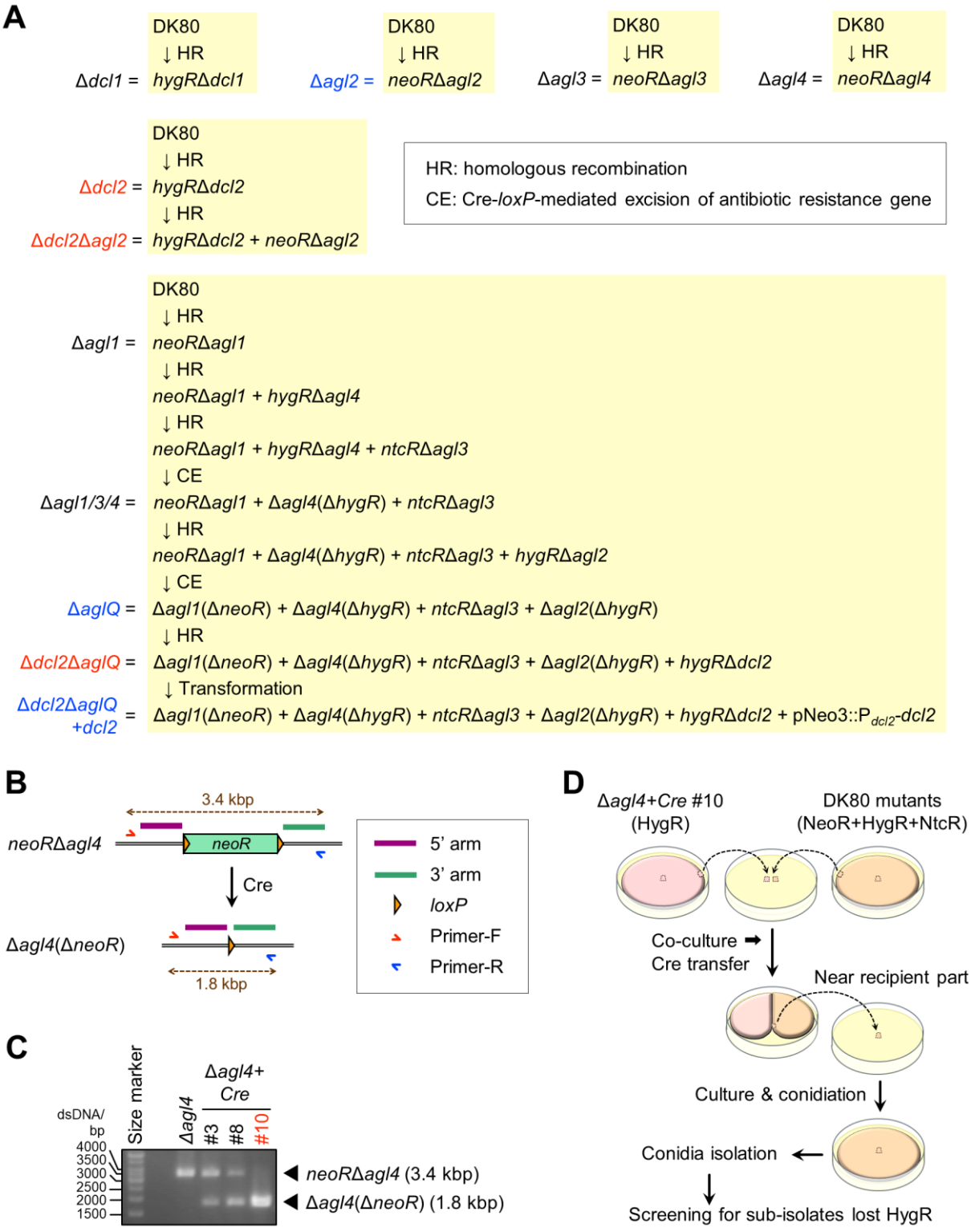


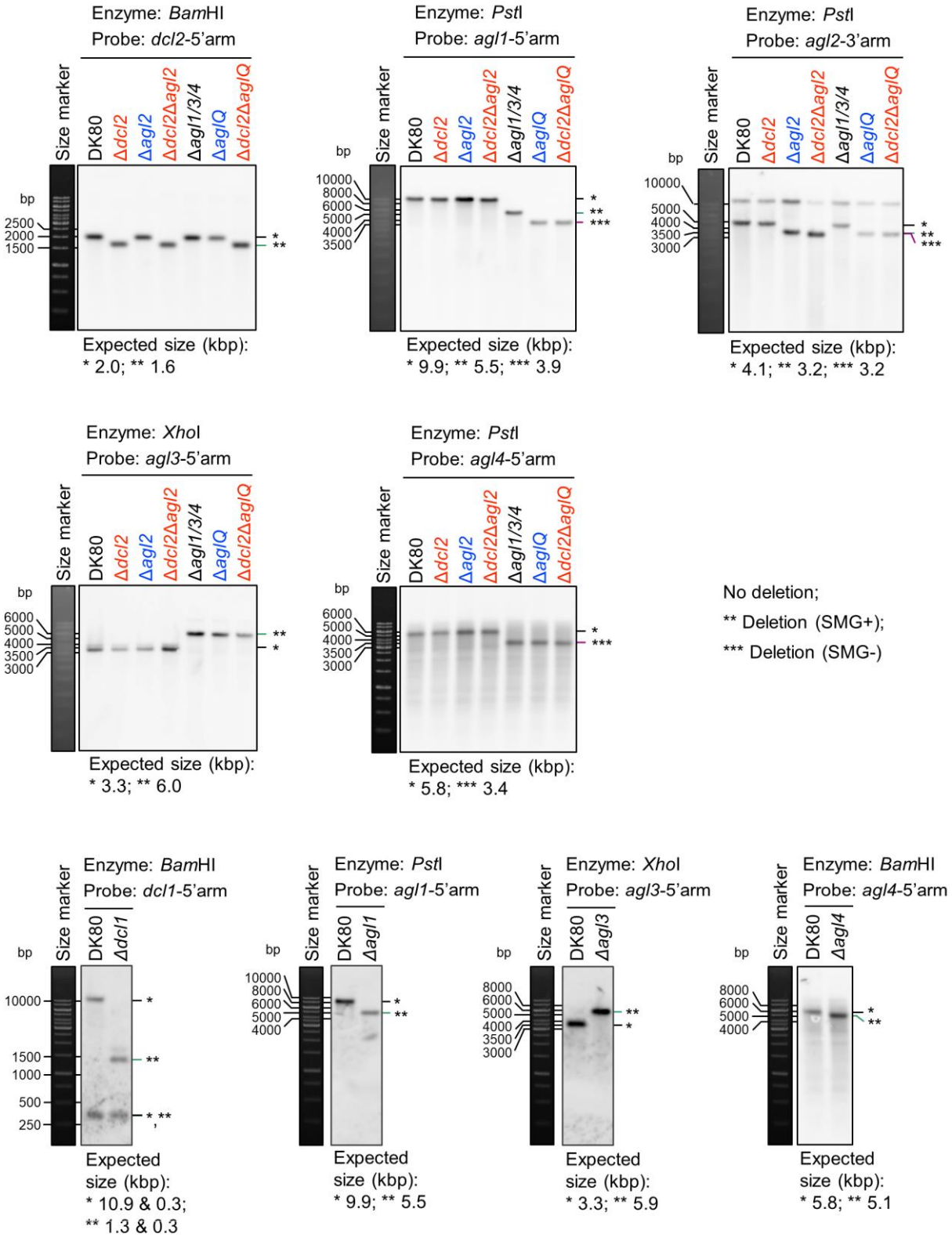
Supplementary Fig. S1. Gene organization and restriction map of wild-type and modified *dcl* and *agl* loci in *Cryphonectria parasitica* DK80.

The organization of wild-type genes is based on a genome assembly of *C. parasitica* strain EP155 (isogenic strain to DK80 except for the *cpku80* locus) (24). The v2.0 unmasked nuclear assembly was searched with *dcl1*, *dcl2*, *agl1*, *agl2*, *agl3*, and *agl4* sequences (*SI Appendix*, Table S4) to obtain the flanking upstream and downstream sequences. The genomic regions are represented by the double lines on which DCL/AGL coding parts (exons) are depicted by the black boxes. The pink two-headed arrows show polymerase chain reaction (PCR) genotyping target regions (Fig. 1*B*). The purple and green boxes indicate the position of the 5′ and 3′ homology arms for gene disruption by homologous recombination. *neoR*, *hygR*, and *ntcR* indicate antibiotic resistance (*R*) gene expression cassettes against neomycin, hygromycin, and nourseothricin, respectively. These antibiotic *R* genes were removed from several gene knockout strains by the Cre-*loxP* system (*SI Appendix*, Fig. S2). The map shows the restriction sites used for Southern blotting (*SI Appendix*, Fig. S3) and the expected fragment length of the probe targets.



Supplementary Fig. S2. Generation of single and multiple *dcl*/*agl* knockout strains in *Cryphonectria parasitica* DK80. (*A*) Steps to generate the single or multiple *dcl*/*agl* gene knockout strains and a *dcl2* complementary strain. *SI Appendix*, Fig. S1 depicts the structure of the modified genes. **(*B*)** Schematic representation for excision of the antibiotic *R* marker gene by Cre-*loxP* system. An example of Δ*agl4* (*neoR*Δ*agl4*) is shown. Cre recombinase excised the antibiotic *R* gene wedged

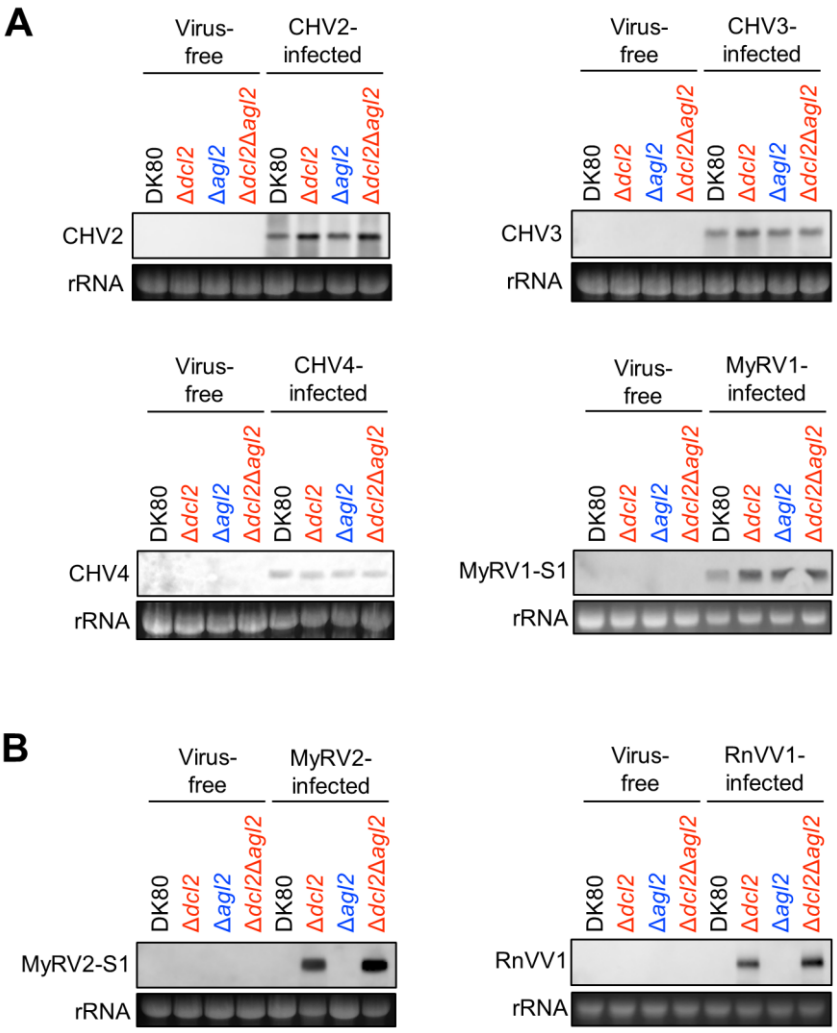
between the *loxP* sequences. **(*C*)** Selection of Δ*agl4+Cre* with high Cre efficacy by polymerase chain reaction (PCR). The PCR targets upstream and downstream of the excised region (***B***) (*SI Appendix*, Table S2) to distinguish the presence or absence of *neoR* by amplicon length. Strain #10 showing complete *neoR* excision was selected as a Cre donor. **(*D*)** The strategy for antibiotic *R* gene excision from the *agl* multiple mutants that show multiple resistance to neomycin, hygromycin, and nourseothricin (NeoR, HygR, and NtcR, respectively), by transiently introducing Cre from dual-cultured Δ*agl4+Cre* #10. Refer to the Methods section for the detailed procedure.



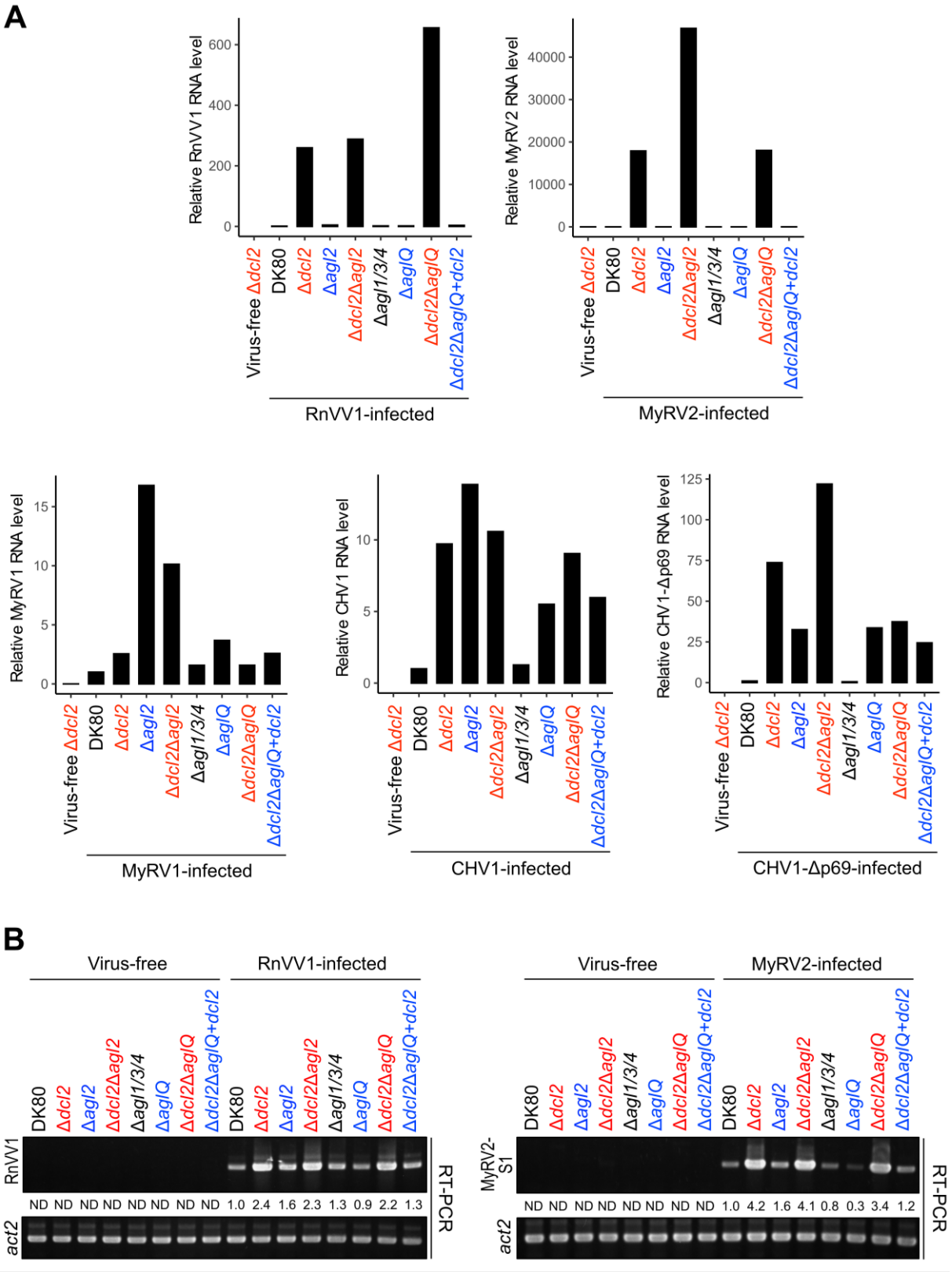
Supplementary Fig. S3. Genotyping of the *dcl*/*agl* knockout strains of *Cryphonectria parasitica* generated in this study. Validation of the targeted genetic modification of Argonaute-like protein genes (*agl1*, *agl2*, *agl3*, and *agl4*) and Dicer-like protein genes (*dcl1* and *dcl2*) by Southern blotting. The probes target the upstream or downstream regions of the deleted regions (*SI Appendix*, Fig. S1). The asterisks indicate the position of the wild haplotype (*), the deletion haplotype carrying the selectable

marker gene (SMG) (**), or another deletion haplotype in which the SMG was removed by the Cre-*loxP* system (***) (*SI Appendix*, Figs. S1 and S2). The Southern hybridization gels were flanked with the size marker stained with ethidium bromide before the blotting.

Supplementary Fig. S4. Colony morphology of the *dcl2/agl* single and multiple knockout strains of *Cryphonectria parasitica*. Virus-free DK80 and its mutants were cultured on potato dextrose agar (9 cm in diameter) for 6 days after a small (1 mm^3) mycelial plug was placed on the center of each plate. The white bar represents 3 cm.

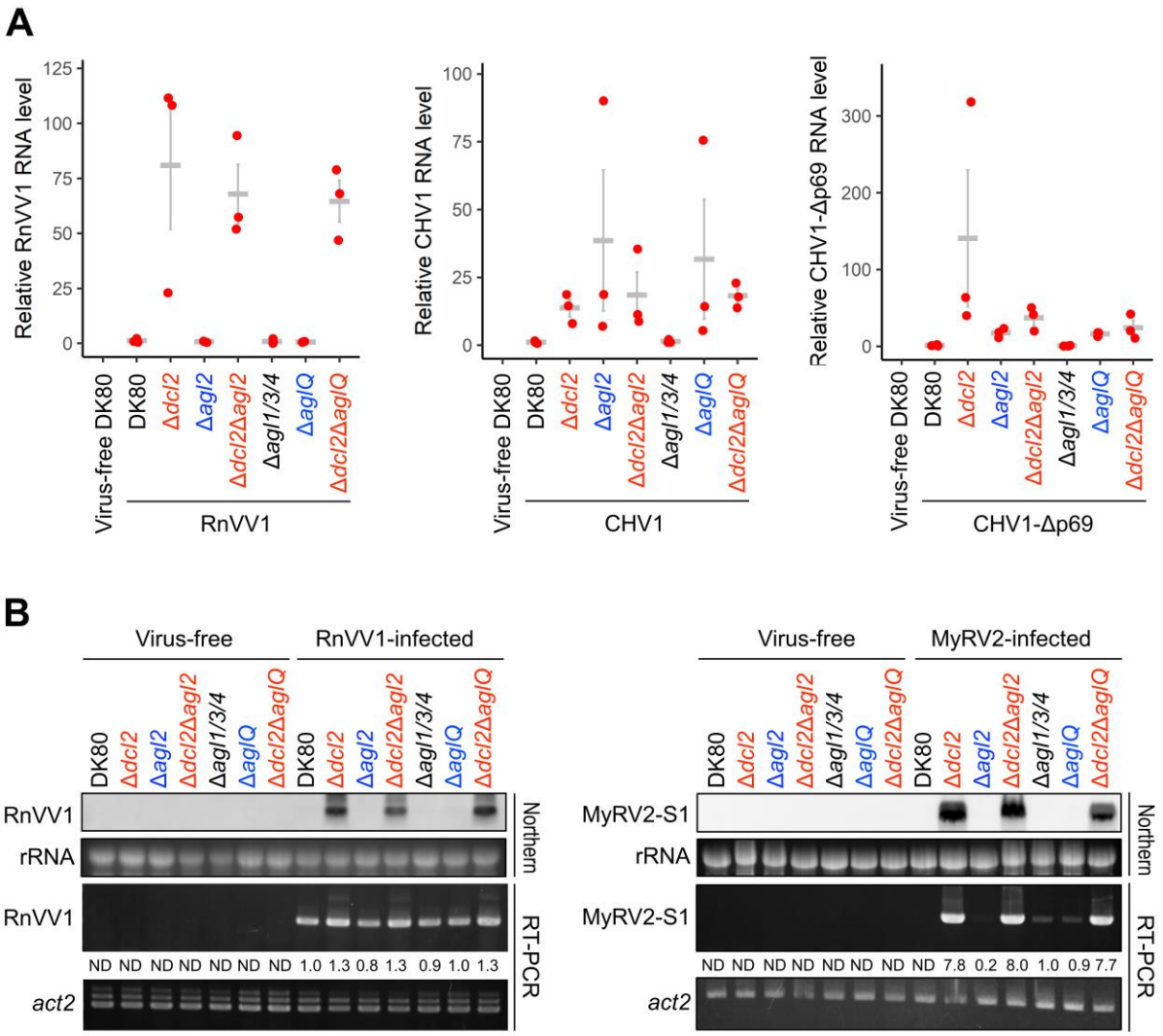


Supplementary Fig. S5. Screening of viruses that are suppressed by *dcl2* but not *agl2*. DK80, Δ*dcl2*, Δ*agl2*, and Δ*dcl2*Δ*agl2* were inoculated with viruses via hyphal fusion (Fig. 2*A*). Viral RNA in the recipients in the absence or presence of the viruses was detected by northern hybridization from crude RNA precipitated with LiCl. Host ribosomal RNA (rRNA) is shown as a loading control. **(*A*)** Viruses less affected by *dcl2* and *agl2*. **(*B*)** Viruses highly affected by *dcl2* but not *agl2*.

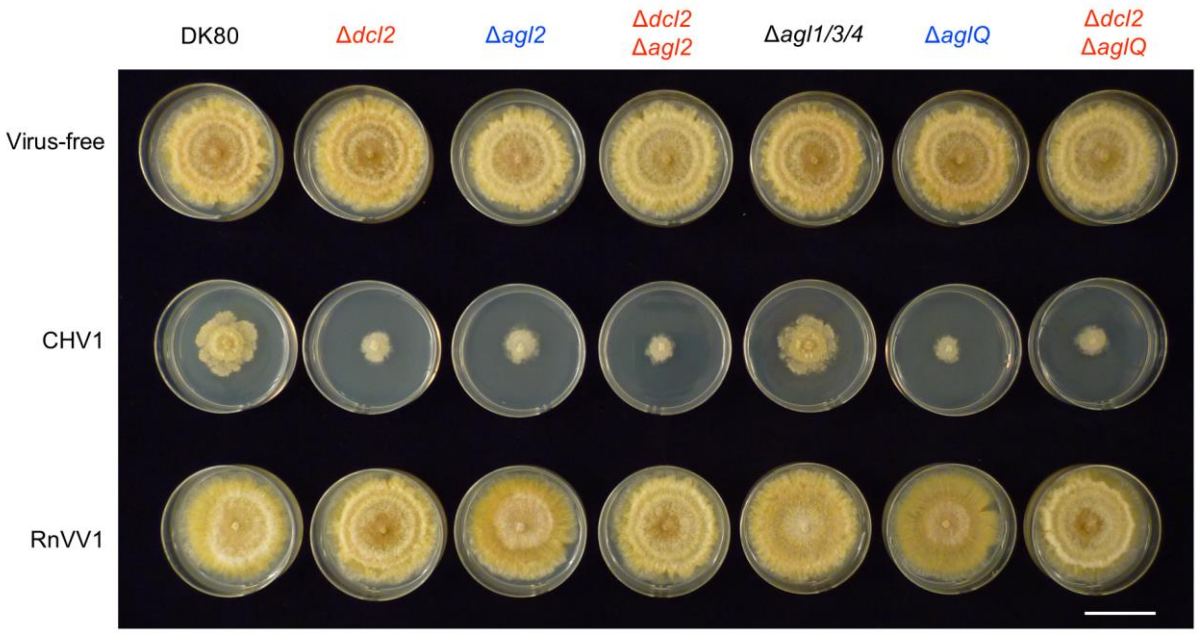


Supplementary Fig. S6. Detection of viral total RNA by quantitative reverse-transcription–polymerase chain reaction (qRT-PCR) and semi-qRT-PCR. The qRT-PCR and semi-qRT-PCR were performed with total RNA (dsRNA + ssRNA) samples used in Fig. 3*A-E*. **(*A*)** qRT-PCR in the fungal strains inoculated with RnVV1, MyRV2, MyRV1, CHV1, or CHV1-Δp69. The bars indicate fold

changes in viral RNA level normalized by the host actin homolog (centractin ortholog) gene expression. The fold changes were calculated based on the value of virus-infected DK80 as 1. **(*B*)** Semi-qRT-PCR in the fungal strains inoculated with RnVV1 or MyRV2. The relative band intensity for semi-qRT-PCR of viral RNA is shown below the electrophoretic image. ND stands for not determined/detected. The host actin homolog gene (*act2*) was analyzed as an internal control for semi-qRT-PCR.



Supplementary Fig. S7. Detection of viral RNA in host ssRNA-enriched nucleic acids by quantitative reverse-transcription–polymerase chain reaction and semi-qRT-PCR. Crude RNA enriched with ssRNA via LiCl was used as a template for cDNA synthesis. **(*A*)** qRT-PCR in the fungal strains inoculated with RnVV1, CHV1, or CHV1-Δp69. The dots indicate fold changes in viral RNA level normalized by the host actin homolog gene expression. The gray cross bars indicate mean values and standard errors in three independent infections. The fold changes were calculated based on the mean value of virus-infected DK80 as 1. **(*B*)** Northern hybridization and semi-qRT-PCR in the fungal strains inoculated with RnVV1 or MyRV2. Host ribosomal RNA (rRNA) is shown as a loading control for northern hybridization. See *SI Appendix*, Fig. S6*B* legend for the details of semi-qRT-PCR.



Supplementary Fig. S8. Viral symptoms in the *dcl2/agl* single and multiple knockout strains of *Cryphonectria parasitica*. Another set of fungal colonies infected by CHV1 and RnVV1 was prepared independently from the Fig. 4 experiment. The white bar represents 3 cm.

Supplementary Table S1. Primers used for vector construction

Construct	Target	F/R [a]	Primer sequence (5'-3') [b]
pGEM::loxPneoR	*neoR*	F	GCGGCCGCGGGAATT**ATAACTTCGTATAATGTATGCTATACGAAGTTAT-**gaattcAGATACCCAGAGTTATCTCACCA
		R	AGGCGGCCGCGAATT**ATAACTTCGTATAGCATACATTATACGAAGTTAT-**gaattcTCATTCCCGGTGTAGGAAGC
pGEM::loxPhygRinv	*hygR*	F	ACGAAGTTATGAATTCAGAAGATGATATTGAAGGAGCA
		R	ACGAAGTTATGAATTtctagaAAGAAGGATTACCTCTAA
pGEM::loxPntcRinv	*ntcR*	F	ACGAAGTTATGAATTCAGAAGATGATATTGAAGGAGCA
		R	TTAAGTGGATGGATCCTCAGGGGCAGGGCATGCTCA
pGEM::loxPhygRinvΔ*dcl1*	*dcl1*-5'arm	F	ATTGGGCCCGACGTCGCATGCTTCGTCGCTGTTCTGTAATACTG
		R	CCATGGCGGCCGGGAGCATGCCTTGGCCTCGATTTGGGACA
	dcl1-3'arm	F	CGCGGCCGCCTGCAGGTCGACCAGCGATCTGAAGAGTTGTG
		R	GAGCTCTCCCATATGGTCGACCGCGTTGAGATACCCTGTTG
pGEM::loxPhygRinvΔ*dcl2*	*dcl2*-5'arm	F	ATTGGGCCCGACGTCGCATGCACTGACTTTCACAGGTCCAT
		R	CCATGGCGGCCGGGAGCATGCCTTGCAGCGTCGTACGACA
	dcl2-3'arm	F	CGCGGCCGCCTGCAGGTCGACGGTTCGGACCGACAAGAATG
		R	GAGCTCTCCCATATGGTCGACATGAGACACTGATCGTCCTT
pGEM::loxPneoRΔ*agl1*	*agl1*-5'arm	F	ATTGGGCCCGACGTCGCATGCCTCTTCTCTGTGGACCGACCTC
		R	CCATGGCGGCCGGGAGCATGCTGTTGACGACGAGTGCTAGATG
	agl1-3'arm	F	CGCGGCCGCCTGCAGGTCGACAGAGCTCTCTTGTCATGACAGC
		R	CAGCTCTCCCATATGGTCGACCCTGGTCACCATGAGGAACTGC
pGEM::loxPneoRΔ*agl2*, pGEM::loxPhygRinvΔ*agl2*	*agl2*-5'arm	F	ATTGGGCCCGACGTCGCATGCCTTTTCTGCGCATTGCTTTCAT
		R	CCATGGCGGCCGGGAGCATGCAGAACCTAGGCGAGACGCGATA

pGEM::loxPneoRΔ*agl1*, pGEM::loxPntcRinvΔ*agl3*	*agl2*-3'arm	F	CGCGGCCGCCTGCAGGTCGACAACTTTGACGGACCATCCTGTC
		R	GAGCTCTCCCATATGGTCGACTTAGCGTTTGTTGTAGTGTTAA
	agl3-5'arm	F	ATTGGGCCCGACGTCGCATGCTTAATTTCATTTACAAGTAGAC
		R	CCATGGGCGGCCGGGAGCATGCGCCACTAATGCTCAACTGGCTG
	agl3-3'arm	F	CGCGGCCGCCTGCAGGTCGACGGTAGGGAGGGTCGAAAGAAAG
		R	CAGCTCTCCCATATGGTCGACGCTACGCCGCCTCCAAGGCCGG
pGEM::loxPneoRΔ*agl1*, pGEM::loxPhygRinvΔ*agl4*	*agl4*-5'arm	F	ATTGGGCCCGACGTCGCATGCTTTCCAGACTGTAGTTTCATTT
		R	CCATGGCGGCCGGGAGCATGCAGGTCCAGAAGAAGCTCCTGCA
	agl4-3'arm	F	CGCGGCCGCCTGCAGGTCGACGACCACCGGCCAGCGAATGGTG
		R	CAGCTCTCCCATATGGTCGACATGCGTATATACCCTGCTGCCT
pCPXHY3::Cre	Cre CDS	F	CAAGCTTGTTAACGCGGCCGCATGTCCAATTTACTGACCGT
		R	GGAGATCAGGTCAAGCATGCCTAATCGCCATCTTCCAGC
pXYLHY3	P_{xyl}	F	GTTTGGGCCAGAGCTactagtctcgagAAACTTGCCGGATTTACCCT
		R	CCGCGTTAACAAGCTTGGGATTGTAAGGGGACGATG
pXYLNeo3	*neoR*	F	TGCTTGGGCCTAATgaattcAGATACCCAGATACCCAGAG
		R	ACACTATAGAATACtctagaTCATTCCCGGTGTAGGAAGC
pNeo3::P_{dcl2}-*dcl2*	P_{dcl2}-*dcl2*	F	AGCTACTAGTCTCGAGGTTTTGCTCGACGTCGATGC
		R	GGAGATCAGGTCAAgcatgcGGAGTTCGACGTATCCATGT

[a] Primer orientation (F: forward; R: reverse).

[b] Underlined, bold, and lowercase letters indicate attached sequences of linearized vector terminal, *loxP*, and restriction site, respectively.

Supplementary Table S2. Primers used for detection of viruses and host genes

Target gene/virus	Accession:position [a]	F/R* [b]	Primer sequence (5'-3')	Amplicon length (bp) [c]
agl4 flanking regions (*SI Appendix*, Fig. S2)	Crypa2|scaffold_6: 301638-306290	F	CCCATATTCATCTTCTGCACC	*neoR*Δ*agl4*: 3440; Δ*agl4*(Δ*neoR*): 1786
		R	(*agl4*-3'arm reverse) [d]	
dcl1 ORF	DQ186989.1:825-1391	F	ATGGGTGATCCAGCGGCG	gDNA: 567
		R	AAGCCACCTTTTCGACCAGG	
dcl2 ORF	DQ186990.1:1569-2146	F	ATGGCGTACTATACCGACTC	gDNA: 597; cDNA: 424
		R	CGCCTGTGACGATTTTGGTC	
agl1 ORF	GQ250184.1:1011-1521	F	TGCACGGGATCATTCAGAGG	gDNA: 511
		R	CGCTGGCATGCAAATCTAATC	
agl2 ORF	GQ250185.1:1908-2724	F	GTATGGCGTCAATCCCAATG	gDNA: 836; cDNA: 682
		R	CACCTTGCTGCTAGGTTTGG	
agl3 ORF	GQ250186.1:2311-2974	F	CTCGAAGAAGTCACCACCAC	gDNA: 664
		R	ATTGAGAGTTCTGTCCCCGT	
agl4 ORF	GQ250187.1:1196-1951	F	CCTCCTGCTCTCGGATATGA	gDNA: 756
		R	AGTTTCGCTTTATGGCAACCAG	
act2 ORF (northern, semi-qRT-PCR)	MU032352.1:558771-559410	F	ATGGCGGATTCACTGCACAA	gDNA: 640; cDNA: 520
		R	TGGCAAACCCCTCGTACACC	
act2 cDNA (qRT-PCR)	XM_040926251.1:622-736	F	GACGTTGAGCGAAGAGCACC	115
		R	GTAGAGAGCGGGGACGTTGA	
CHV1 (northern)	M57938.1:9157-9646	F	TGTACACCTCGTGTGAACGG	490
		R	TGAGTGTGCCACGGATTCTC	
CHV1 and CHV1-Δp69 (qRT-PCR)	M57938.1:9543-9646	F	ATAGTGGACGGGGTGTATCG	104
		R	TGAGTGTGCCACGGATTCTC	

CHV1-Δp69 (northern)	M57938.1:3265-3764	F	TGGAAGAGGCGAAAGAGGCA	500
		R	TGTCGAAGGGTTGGTATTAT	
RnVV1 (northern, semi-qRT-PCR)	AB742454.1:3970-4556	F	TCAACAGTCACCACTCGACG	606
		R	TTGATTTCCCGTGGCGTACA	
RnVV1 (qRT-PCR)	AB742454.1:4441-4575	F	TCAACCCCAACCCGATGACA	135
		R	TTGATTTCCCGTGGCGTACA	
MyRV1-S1 (northern)	AY277888.1:2662-3112	F	AACTTTCGCCCTACGCATGA	451
		R	TGGCGAGAGTTCCATTGCAT	
MyRV1-S1 (qRT-PCR)	AY277888.1:3004-3112	F	CGATTCAAGTGCTACGGCAT	109
		R	TGGCGAGAGTTCCATTGCAT	
MyRV2-S1 (northern)	See legend [e]	F	GGTTTCTCCCCGAGATAGCG	537
		R	AGGTTCTTACAGCTGACCGC	
MyRV2-S1 (qRT-PCR)	See legend [e]	F	GCAATATGCTCACCGTGGTA	135
		R	AGGTTCTTACAGCTGACCGC	
CHV2	L29010.1:3138-3590	F	CATTTCCGAAGAGCCATGCG	453
		R	CCGAATTCCAAGCGCATCAG	
CHV3	AF188515.1:2045-2082	F	AGAGGGTTATGCCCCCTGAT	538
		R	ATCTGCCATCTATCAGCCGC	
CHV4	MK533145.1:287-875	F	ATGTCTGAGCAACAACTCATCT	589
		R	TGCCATCCACCAGATGCCAGTT	

[a] The accession number in JGI Genome Portal is shown for "*agl4* flanking regions", while that in GenBank is listed for the others.

[b] Primer orientation (F: forward; R: reverse).

[c] gDNA: genomic DNA of *C. parasitica*; cDNA: complementary DNA reverse transcribed from messenger RNA of *C. parasitica* or viruses.

[d] Primer sequence is included in *SI Appendix*, Table S1.

[e] Personally provided by Dr. Bradley I. Hillman (Rutgers University).

Supplementary Table S3. Viral and fungal strains used in this study

Strain	Description	Reference
Viral		
CHV2	Capsidless non-segmented (+)RNA virus in the genus *Hypovirus* in the family *Hypoviridae*, from *C. parasitica* strain NB58	(31)
CHV3	Capsidless non-segmented (+)RNA virus in the genus *Hypovirus* in the family *Hypoviridae*, from *C. parasitica* strain GH2	(32)
CHV4	Capsidless non-segmented (+)RNA virus in the genus *Hypovirus* in the family *Hypoviridae*, from *C. parasitica* strain C18	(33)
Fungal		
EP155/CHV2	*C. parasitica* EP155 inoculated with CHV2 isolate NB58	(34)
EP155/CHV3	*C. parasitica* EP155 inoculated with CHV3 isolate GH2	(34)
EP155/CHV4	*C. parasitica* EP155 inoculated with CHV4 isolate C18	This study
Δagl4+Cre	*C. parasitica* DK80*Δagl4* transformed with pCPXHY3::Cre	This study

Supplementary Table S4. ID of RNAi genes in *C. parasitica* EP155/DK80.

Gene name	GenBank accession	Gene ID in Crypa2 [a]	Transcript ID in Crypa2 [a]	Reference
rdr1	HF912382	e_gw1.11.21.1	270014	(35)
rdr2	HF912383	Crypa1.e_gw1.1.1699.1	35624	
rdr3	HF912384	Crypa1.gw1.4.33.1	10929	
rdr4	HF912385	fgenesh1_pm.4_#_888	339656	
dcl1	DQ186989	Crypa1.fgenesh1_pg.C_scaffold_3001033	69967	(36)
dcl2	DQ186990	estExt_Genewise1.C_41219	276559	
agl1	GQ250184	Crypa1.fgenesh1_pg.C_scaffold_9000048	74333	(4)
agl2	GQ250185	estExt_Genewise1Plus.C_60308	292762	
agl3	GQ250186	e_gw1.9.51.1	268359	
agl4	GQ250187	e_gw1.6.1921.1	261854	

[a] *Cryphonectria parasitica* EP155 v2.0 genome assembly (https://mycocosm.jgi.doe.gov/Crypa2) (24).