Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection

Ming Wang¹, Arne Weiberg¹, Feng-Mao Lin², Bart P. H. J. Thomma³, Hsien-Da Huang² and Hailing Jin¹*

Aggressive fungal pathogens such as Botrytis and Verticillium spp. cause severe crop losses worldwide. We recently discovered that Botrytis cinerea delivers small RNAs (Bc-sRNAs) into plant cells to silence host immunity genes. Such sRNA effectors are mostly produced by Botrytis cinerea Dicer-like protein 1 (Bc-DCL1) and Bc-DCL2. Here we show that expressing sRNAs that target Bc-DCL1 and Bc-DCL2 in Arabidopsis and tomato silences Bc-DCL genes and attenuates fungal pathogenicity and growth, exemplifying bidirectional cross-kingdom RNAi and sRNA trafficking between plants and fungi. This strategy can be adapted to simultaneously control multiple fungal diseases. We also show that Botrytis can take up external sRNAs and double-stranded RNAs (dsRNAs). Applying sRNAs or dsRNAs that target Botrytis DCL1 and DCL2 genes on the surface of fruits, vegetables and flowers significantly inhibits grey mould disease. Such pathogen gene-targeting RNAs represent a new generation of environmentally friendly fungicides.

SRNAs are short non-coding regulatory RNAs that are mostly generated by Dicer or Dicer-like (DCL) endoribonucleases from dsRNAs or single-stranded RNAs (ssRNAs) with hairpin structures¹-². Mature sRNAs are loaded into Argonaute (AGO) proteins and induce silencing of genes with fully or partially complementary sequences³-⁴. This phenomenon is called RNA interference (RNAi) and is widely conserved in most eukaryotic organisms. We have recently discovered that B. cinerea, an aggressive plant fungal pathogen, delivers sRNAs (Bc-sRNAs) into host plant cells and utilizes host RNAi machinery to suppress host immunity genes⁵. These pathogen sRNAs mediate cross-kingdom RNAi in plant hosts and represent a novel class of pathogen effectors that inhibit host immunity for successful infection⁶-⁷. Similarly, some animal parasites, such as gastrointestinal nematode Heligmosomoides polygyrus, also deliver sRNAs into mammalian cells and target host genes involved in innate immunity⁸-¹². These studies suggest that sRNA-mediated cross-kingdom RNAi is a conserved virulence mechanism that evolved in both plant and animal eukaryotic pathogens and pests to suppress host immunity. However, it is still not clear how many pathogens and pests have evolved this novel pathogenic mechanism.

While we have demonstrated sRNA trafficking from fungal pathogens to host plants, others have observed that plant transgene-derived artificial sRNAs can induce gene silencing in certain interacting insects¹³, nematodes¹⁵,¹⁶, fungi¹⁷,¹⁸ and oomycetes¹⁹,²⁰, a phenomenon called host-induced gene silencing (HIGS)²¹, suggesting that the artificial sRNAs travel from host plants to pathogens and pests. However, bidirectional cross-kingdom RNAi and two-way sRNA trafficking have not been observed between a pathogen and a host, or any two interacting organisms.

Plant diseases triggered by eukaryotic pathogens, such as fungi and oomycetes, cause significant crop losses every year. B. cinerea poses a serious threat to almost all vegetables and fruits, as well as many flowers, in their pre- and post-harvest stages by causing grey mould disease²²,²³. Verticillium dahiae is another economically important fungal pathogen, which causes wilt disease on a wide range of plant species²⁴,²⁵. Current disease control is mainly achieved by fungicide application, which is costly and environmentally hazardous. HIGS has worked effectively against certain fungal and oomycete pathogens, such as Blumeria graminis²⁶,²⁷, Puccinia triticii²⁸, Fusarium spp.²⁹,³⁰, Phytophthora infestans³¹ and Phytophthora capsici³², but it is restricted to plants with established transformation methods, which are not available to the majority of crop plants. Moreover, genetically modified organisms (GMOs) are still a concern to some consumers. Thus, there is an urgent need to develop effective solutions that are environmentally friendly to control plant diseases caused by pathogens that utilize sRNA effectors, such as B. cinerea.

Results

To explore whether cross-kingdom RNAi is bidirectional in B. cinerea—plant interactions and to find a way to protect plants from grey mould disease, we first reasoned that generating HIGS plants producing artificial, traceable sRNAs that target virulence genes in B. cinerea would be a straightforward approach. The B. cinerea genome encodes two DCL genes, Bc-DCL1 and Bc-DCL2 (Bc-DCL1/2). We previously showed that the pathogenicity and growth of B. cinerea were largely compromised when both DCLs were knocked out³. To fully evaluate the contribution of Bc-DCLs to B. cinerea pathogenicity on a wide range of economically important crops, we inoculated various fruits (tomato, Solanum lycopersicum ‘Roma’; strawberry, Fragaria × ananassa; and grape, Vitis labrusca ‘Concord’), vegetables ( iceberg lettuce, Lactuca sativa; and onion, Allium cepa L.) and flower petals (rose, Rosa hybrida L.) with the dcl1 dcl2 double mutant, dcl1 and dcl2 single mutants and wild-type (WT) strains. Only the dcl1 dcl2 double mutant, but not the dcl1 or dcl2 single mutants, showed much weaker pathogenicity and produced significantly smaller lesions than the WT strain on all the plant samples (Fig. 1a,b, P < 0.01), although all the mutant strains showed reduced growth in planta (Fig. 1c) and on cultured media.

¹Department of Plant Pathology and Microbiology, Center for Plant Cell Biology, Institute for Integrative Genome Biology, University of California, Riverside, California 92521, USA. ²Institute of Bioinformatics and Systems Biology, National Chiao Tung University, Hsin-Chu 300, Taiwan. ³Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, the Netherlands. ⁴Present address: Institute of Genetics, University of Munich, Martinsried, Germany. *e-mail: hailing@ucr.edu
More importantly, only the dcl1 dcl2 double mutant, but not the dcl1 or dcl2 single mutants, failed to produce Bc-sRNA effectors\(^5\). Thus, elimination of Bc-sRNA effectors contributes more to the reduced virulence seen in dcl1 dcl2 than growth attenuation, highlighting the importance of both Bc-DCLs and sRNA effectors in fungal pathogenicity. We profiled total sRNAs isolated from the dcl1 dcl2 double mutant and the WT strain. In the WT strain, Bc-sRNAs ranged from 20 to 35 nucleotides (nt) in length (Supplementary Fig. 1b), with an enrichment of 24–27 nt species. The abundance of 20–27 nt sRNA species was clearly reduced but not completely eliminated in the dcl1 dcl2 double mutant (Supplemental Fig. 1b), pointing to the existence of DCL-independent sRNA biogenesis pathways in B. cinerea, as reported in Neurospora crassa\(^32,33\). Most strikingly, the sRNAs derived from most long terminal repeats (LTRs) retrotransposons (ranging from 20–26 nt and peaking at 21–22 nt) were almost completely eliminated in the dcl1 dcl2 double mutant (Supplementary Fig. 1b), which indicates that Bc-DCL1/2 are responsible for generating almost all sRNAs from retrotransposons. The sRNAs from intergenic non-coding (IGN) regions (mainly the 21, 22 and 24 nt sRNAs) and

---

**Figure 1** | B. cinerea dcl1 dcl2 double mutant, but not the dcl1 or dcl2 single mutants, displays reduced virulence on fruits, vegetables, and flower petals.  
**a**, B. cinerea dcl1 dcl2 double mutant shows compromised virulence on fruits (tomato, strawberry and grape), vegetables (lettuce and onion) and flower petals (rose), and B. cinerea dcl1 and dcl2 single mutants showed similar virulence as the WT strain. **b**, Relative lesion sizes of the infected plant samples were measured 3 days post inoculation (dpi) for lettuce, onion and strawberry and 5 dpi for tomato, grape and rose petal using ImageJ. Error bars indicate the standard deviations (s.d.) of ten samples. **c**, B. cinerea relative DNA content (relative biomass) was measured by quantitative PCR. Error bars indicate the s.d. of three technical replicates. Asterisks indicate statistically significant differences (\(P < 0.01\)). Similar results were obtained from at least three biological replicates.
ance to open reading frames (ORFs-antisense, mainly the 21–22 nt sRNAs) were also largely reduced in dcl1 dcl2, although the sRNAs from the sense transcripts of ORFs (ORFs-sense) were not changed significantly. This result is consistent with our previous findings that the majority of predicted sRNA effectors are from retrotransposon LTRs. Bc-DCL1/2 are largely responsible for generating sRNA effectors (Supplementary Table 1) and contribute significantly to B. cinerea pathogenicity. Although it is not known whether HIGS works in B. cinerea, these results show that Bc-DCL1/2 are ideal targets for testing whether HIGS would be an efficient strategy for controlling grey mould disease and whether cross-kingdom RNAi in the opposite direction from the plant host to B. cinerea occurs.

We generated transgenic Bc-DCL1/2–RNAi Arabidopsis plants expressing hairpin RNAs containing two segments from non-conserved regions of Bc-DCL1 and Bc-DCL2, respectively. We avoided the conserved functional domains to eliminate any off-target effects on DCL genes in plants or other organisms. These selected Bc-DCL DNA regions have only 3.5–4.8% sequence homology to Arabidopsis DCLs (Supplementary Fig. 2a), and, indeed, no host DCL genes were silenced (Supplementary Fig. 2b). The hairpin RNA products in the transgenic plants were processed into sRNAs (Bc-DCL1/2–sRNAs) (Fig. 2a). These RNAi plants exhibited much smaller lesions and less fungal growth after B. cinerea infection than the WT plants (Fig. 2b,c). Relative expression of Bc-DCL1 and Bc-DCL2 was clearly suppressed in B. cinerea collected from the transgenic plants compared with those from WT plants (Fig. 2d).

These results suggest that RNAi signals against Bc-DCL1/2 produced in plant cells moved into the fungal cells and efficiently silenced Bc-DCL1 and Bc-DCL2, which led to suppression of fungal virulence and growth and inhibition of disease.

To determine whether this Bc-DCL1/2-targeting RNAi strategy could also efficiently control grey mould disease in tomato plants, we introduced the same Bc-DCL1/2 RNAi fragment into the tobacco rattle virus (TRV) silencing system, which triggered the expression of Bc-DCL1/2–sRNAs in tomato. Three weeks after agro-infiltration, the tomato plants produced large amounts of Bc-DCL1/2–sRNAs (Fig. 2e) and were infected with B. cinerea using spray inoculation. The tomato leaves expressing Bc-DCL1/2–sRNAs displayed very mild to almost no disease symptoms at 3 dpi (Fig. 2f,g), whereas the controls, which either expressed sRNAs targeting a potato late blight resistance gene RB that is not present in tomato or the TRV2 empty vector (EV), both showed very severe water-soaked disease lesions (Fig. 2f,g). The growth of B. cinerea was also significantly reduced on the leaves expressing Bc-DCL1/2–sRNAs compared with the controls, and the expression of Bc-DCL1 and Bc-DCL2 was largely reduced in B. cinerea grown on these leaves as well (Fig. 2h). These data further support that cross-kingdom RNAi is bidirectional in B. cinerea—plant interactions, and fungal DCLs are ideal target genes to knock down in order to control pathogens that use sRNA effectors.

B. cinerea also infects many other plant species in addition to Arabidopsis and tomato. Grey mould disease is a very serious problem in post-harvest management as it destroys millions of fruits, vegetables and flowers during the packing, transportation and storage processes each year. Plant protection provided by cross-kingdom RNAi or HIGS is effective in some plant–pathogen interactions. However, this method is restricted to plants with established transformation protocols, but the majority of crop plants do not have available genetic manipulation tools. Moreover, GMOs are still a concern to many consumers. Therefore, we were motivated to explore whether RNA-based plant protection against eukaryotic pathogens can be achieved through a non-GMO approach. Uptake of RNAs from the environment, a phenomenon named environmental RNAi, was observed in C. elegans and certain insects. Spraying dsRNAs targeting the actin gene of potato beetle Leptinotarsa decemlineata on potato leaves inhibited larval growth. In vitro treatment of fungal pathogen F. graminearum with dsRNAs targeting CYP51 genes also inhibited fungal growth, but there is no direct evidence demonstrating environmental dsRNAs move into fungal cells to silence fungal CYP51 genes. Overall, it is unclear whether environmental RNAi works in fungi, plants or mammals.

To investigate whether fungi can take up RNAs from the environment, we synthesized Fluorescein-labelled Bc-DCL1/2–dsRNAs by in vitro transcription using Fluorescein-12-UTP, and we generated Fluorescein-labelled Bc-DCL1/2–sRNAs by RNase III in vitro processing of the Fluorescein-labelled dsRNAs. These dsRNAs and sRNAs were applied on B. cinerea spores (Fig. 3a) and grown on agar medium to determine whether fungal cells could take up RNAs. Fluorescence signals accumulated within fungal cells 12 h after incubation (Fig. 3a). To exclude the possibility that fluorescent RNAs adhered to the exterior site of the fungal cells, we treated the fungus with Micrococcal nuclease (MNase). The fluorescence signals remained detectable (Fig. 3a), indicating that B. cinerea cells took up the dsRNAs and sRNAs. Fluorescein-12-UTP and water treatment were used as negative controls. To determine whether there is fungal preference for a type of RNAs or RNAs with specific sequences, we also tested Fluorescein-labelled YFP–sRNAs and YFP–dsRNAs. These RNAs were also efficiently taken into the fungal cells, suggesting that such RNA uptake is not likely to be selective. To confirm that the RNAs entered the cytoplasm of B. cinerea and were not buried in the cell wall matrix, fluorescent RNAs were added to a liquid culture of B. cinerea germinating spores (Fig. 3b), which were subjected to protoplast preparation to remove all the cell walls after 20 h of incubation. The fluorescence signal was clearly detected within B. cinerea protoplasts even after MNase treatment (Fig. 3b).

It is worth noting that the fluorescence intensities of labelled sRNAs were lower than that of dsRNAs (Supplementary Fig. 3). Furthermore, B. cinerea treated with Bc-DCL1/2–dsRNAs or –sRNAs clearly showed reduced Bc-DCL1 and Bc-DCL2 mRNA levels (Fig. 3c). Thus, fungal cells can take up dsRNAs and sRNAs from the environment, which then induces gene silencing in the fungal cells.

Moreover, after entering WT B. cinerea cells, Bc-DCL1/2–dsRNAs were processed into Bc-DCL1/2–sRNAs by Bc-DCLs, but Bc-DCL1/2–sRNAs were not detected in the dcl1 dcl2 mutant (Fig. 3d). This provided an excellent system to determine whether sRNAs are mobile silencing signals transported from the plant into B. cinerea in the HIGS systems. Although various RNAi mobile signals, including sRNAs and dsRNA precursors, in HIGS have been proposed, none has been clearly confirmed. In C. elegans, Drosophila and western corn rootworm (WCR, Diabrotica virgifera virgifera LeConte), it is the long dsRNAs instead of sRNAs that are taken up efficiently. Transgenic Arabidopsis expressing hairpin Bc-DCL1/2–RNAi, which showed a high level of processed Bc-DCL1/2–sRNAs (Fig. 2a), were inoculated with the dcl1 dcl2 mutant that was unable to process Bc-DCL1/2–dsRNAs into Bc-DCL1/2–sRNAs (Fig. 3d). At 48 hours post inoculation, we managed to isolate pure B. cinerea protoplasts from plant cells via sequential protoplast preparation by taking advantage of the different cell wall compositions between plants and fungi. The mock-treated Arabidopsis control mixed with B. cinerea dcl1 dcl2 mycelium before protoplast isolation was used as a control of the procedure to monitor the purity of the isolated B. cinerea protoplasts. Bc-DCL1/2–sRNAs were detected in the B. cinerea dcl1 dcl2-infected Arabidopsis sample but not in the control (Fig. 3e), supporting that Bc-DCL1/2–sRNAs indeed translated into B. cinerea. The Bc-DCL-independent sRNA Bc-siR1498 that we previously identified was used as an internal control to show the amount of B. cinerea in each sample of the experiments. Thus, sRNAs are at least one of the major mobile signals for cross-kingdom RNAi in HIGS. We do not rule out that other forms of RNAs, such as long dsRNAs, could also be transported; however,
**Figure 2** | Arabidopsis and tomato Bc-DCL1/2–RNAi plants confer enhanced resistance against *B. cinerea* infection. **a**, Bc-DCL1/2–sRNAs were highly expressed in the Arabidopsis transgenic plants, as detected by northern blot. **b**, Arabidopsis Bc-DCL1/2–RNAi plants show enhanced disease resistance against *B. cinerea*. **c**, Relative lesion sizes were measured at 3 dpi using ImageJ. Error bars indicate the s.d. of ten samples. *B. cinerea* relative biomass was measured at 3 dpi by quantitative PCR. Error bars indicate the s.d. of three technical replicates. **d**, The expression of Bc-DCL1 and Bc-DCL2 were downregulated in infected Arabidopsis Bc-DCL1/2–RNAi plants, as measured by quantitative RT–PCR. **e**, Northern blot analysis reveals the expression levels of Bc-DCL1/2–sRNAs in the tomato Bc-DCL1/2–RNAi plants through virus-induced gene silencing (VIGS). **f**, Tomato Bc-DCL1/2–RNAi plants were more resistant to *B. cinerea* than control plants (EV and RB). Similar results were obtained from three biological replicates. **g**, B. cinerea relative biomass was measured at 3 dpi; error bars indicate the s.d. of three technical replicates. **h**, Bc-DCL1 and Bc-DCL2 were silenced in infected tomato Bc-DCL1/2–RNAi plants, as measured by quantitative RT–PCR. Asterisks indicate statistically significant differences (*P* < 0.01). Similar results were obtained from three biological replicates.
they accumulated to a much lower level than sRNAs in planta, because most of the hairpin dsRNA precursors are efficiently processed into sRNAs.

Since environmental RNAi can be achieved in fungi, we attempted to externally apply synthetic Bc-DCL1/2–sRNAs or Bc-DCL1/2–dsRNAs on the surface of various plants, to test...
whether *B. cinerea* is capable of taking up these dsRNAs and/or sRNAs from the environment and inducing silencing of its own genes. The same *Bc-DCL1/2* RNAi fragment was transcribed *in vitro* from both ends and gave rise to *Bc-DCL1/2*–dsRNAs (Fig. 4a). The *Bc-DCL1/2*–dsRNAs were subjected to RNAse III treatment to generate *Bc-DCL1/2*–sRNAs *in vitro* (Fig. 4a). The *Bc-DCL1/2*–sRNAs and the precursor dsRNAs (20 ng µl⁻¹ in pure water) were externally applied on fruits (tomato, strawberry and grape), vegetables (lettuce and onion) and flower petals (rose) compared with the treatments using water, *YFP*–dsRNAs and –sRNAs. c. The relative lesion sizes and fungal biomass were measured at 3 dpi for lettuce, onion, rose and strawberry and at 5 dpi for tomato and grape fruits using ImageJ software and quantitative PCR, respectively. Error bars indicate the s.d. of ten samples and three technical repeats for the relative lesion sizes and fungal biomass, respectively. Asterisks indicate statistically significant differences (*P* < 0.01). Similar results were obtained from three biological replicates.
RNAs seems to provide stronger protection (Fig. 4bc and Supplementary Fig. 5). We also examined the duration of plant protection against grey mould disease by inoculating the plant samples with *B. cinerea* 1, 3, and 5 days after RNA treatment, and pictures were taken 3 days after the fungal inoculation (dpi) for rose petals (6–8 days after RNA treatment), and 5 dpi for tomato (8–10 days after RNA treatment). We found that the RNAs remained effective for at least up to 8–10 days after RNA treatment, although plant protection was clearly reduced at later time point (Supplementary Fig. 6). It is worth noting that most fungicides contain various adjuvants, which help stabilize the active compounds, although we only used RNAs dissolved in water. Mixing the RNAs with reagents that stabilize RNAs is likely to extend their effective period. We would pursue this in a follow-up study with a more applied focus.

Although external treatment of Bc-DCL1/2–sRNAs and the precursor Bc-DCL1/2–dsRNAs can inhibit fungal disease, *in vitro* RNA synthesis is too costly. To obtain a large amount of Bc-DCL1/2–sRNAs and –dsRNAs at a much lower cost, we expressed the Bc-DCL1/2–RNAi construct in *N. benthamiana*, which yielded a large amount of Bc-DCL1/2–sRNAs, but with a low level of Bc-DCL1/2–dsRNAs and intermediate processing products (Fig. 5a and Supplementary Fig. 7). The plants expressing the Hellsgate EV or the YFP–RNAi construct, which also yielded a large quantity of YFP–sRNAs and a very low level of YFP–RNAs with longer lengths, were used as controls. The purified total RNAs were sprayed onto the surface of fruits, vegetables, and rose petals, followed with *B. cinerea* infection. All the plants treated with the RNA extracts from Bc-DCL1/2–RNA plants developed less severe disease symptoms and showed decreased fungal growth compared with the RNA extracts from plants expressing EV or YFP–RNAs (Fig. 5b–d). This result demonstrates that the Bc-DCL1/2–RNAs, but not *N. benthamiana* total RNAs, can protect plants from *B. cinerea* infection.

Such an RNAi-based disease control method could be powerful if it is effective against multiple pathogens. For example, we could design DCL-targeting dsRNAs or sRNAs to silence DCL genes of multiple pathogens that utilize sRNA effectors. To prove the concept, we chose to examine the soil-borne fungal pathogen *V. dahliae* that displayed reduced virulence on *Arabidopsis*
into host AGO1 protein during infection, we performed RNAi to suppress host immunity genes by hijacking host AGO1.

Figure 6 | Arabidopsis plants expressing hairpin RNAs that simultaneously target DCL genes of B. cinerea and V. dahliae show enhanced disease resistance to both pathogens. a, The Arabidopsis ago1-27 mutant was more resistant to V. dahliae than WT plants in root culture conditions. b, Arabidopsis ago1-27, but not ago2-1 mutant, was less susceptible to V. dahliae than WT plants grown in soil. c, The expression levels of Bc-DCL1/2-sRNAs and Vd-DCL1/2-sRNAs in the Arabidopsis Bc + Vd-DCLs-RNAi transgenic plants were examined by northern blot analysis. d, Arabidopsis Bc + Vd-DCLs-RNAi plants display reduced disease after infection of B. cinerea. Relative lesion sizes were measured 3 dpi using ImageJ, and error bars indicate the s.d. of three technical replicates. e, Quantitative RT-PCR showed that Bc-DCL1 and Bc-DCL2 were silenced in B. cinerea that from infected Arabidopsis Bc + Vd-DCLs-RNAi plants compared with that from WT plants. f, Arabidopsis Bc + Vd-DCLs-RNAi plants were less susceptible to V. dahliae than WT plants. Relative biomass of V. dahliae was measured at 3 weeks after inoculation. Error bars indicate the s.d. of three technical replicates. g, The expression levels of Vd-DCL1 and Vd-DCL2 were suppressed in V. dahliae that from infected Arabidopsis Bc + Vd-DCLs-RNAi plants. Asterisks represent statistically significant differences (P < 0.01). Similar results were obtained from three biological replicates (a–g).

ago1-27 mutant[7], just like B. cinerea. V. dahliae is another economically important fungal pathogen that causes wilt disease on many plant species, including herbaceous annuals, perennials and woody species[24,25]. So far, there is no effective control method other than toxic fungicide application.

To investigate whether V. dahliae also utilizes cross-kingdom RNAi to suppress host immunity genes by hijacking host AGO1 for its virulence, and whether V. dahliae sRNAs are indeed loaded into host AGO1 protein during infection, we performed RNA immunoprecipitation (RIP) assay on V. dahliae-infected Arabidopsis roots using Arabidopsis AGO1 and AGO2 antibodies as described previously[38,49]. Arabidopsis ago1-27 mutant is less susceptible to V. dahliae than WT plants in both soil and root culture conditions (Fig. 6a,b). The ago2-1 mutant shows no difference compared with WT plants, suggesting that AtAGO2 is not involved in the gene regulation during plant–V. dahliae interaction (Fig. 6b). Therefore, we used AGO2-RIP as a control. Using a stringent target prediction program and 100 reads per million (RPM) total Vd-sRNAs as a cutoff[7], we found that 99 AtAGO1-associated Vd-sRNAs have Arabidopsis target genes, whereas only 13 AtAGO2-associated Vd-sRNAs have predicted Arabidopsis targets (Supplementary Tables 2 and 3). These results suggest that V. dahliae also utilizes sRNA effectors and cross-kingdom RNAi for successful infection; thus, targeting Vd-DCL genes could also be a potential strategy for controlling verticillium wilt disease.

We generated Arabidopsis transgenic plants that express hairpin RNAs targeting DCL genes of both V. dahliae and B. cinerea to test whether the RNAi-based method can simultaneously control both fungal diseases. V. dahliae also has two DCL genes. Again, we were not able to find a DNA region outside the conserved domains that has sufficient homology between Vd-DCL1 and Vd-DCL2 (Vd-DCL1/2) or between the DCLs of V. dahliae and B. cinerea to silence two or more DCLs. Thus, we had to select two DNA segments, each from Vd-DCL1 and Vd-DCL2, which had 2.2–3.1% identity to the four At-DCLs (Supplementary Fig. 8a), and fused them to the fragments of Bc-DCL1 and Bc-DCL2 for RNAi vector construction. These transgenic plants expressed high levels of both Bc-DCL1/2-sRNAs and Vd-DCL1/2-sRNAs (Fig. 6c). As expected, the expression of
Arabidopsis DCL genes was not affected in these transgenic lines (Supplementary Fig. 8b). Pathogen infection assays revealed that these transgenic plants are indeed more resistant to both B. cinerea and V. dahliae (Fig. 6d–g). To determine whether the silencing effect is indeed gene specific, we infected Bc-DCL1/2-RNAi plants with V. dahliae. These plants only express Bc-DCL1/2-2-3-sRNAs but not Vd-DCL1/2-sRNAs. They displayed severe wilt disease symptoms just like the WT plants (Supplementary Fig. 9a, b), indicating that Bc-DCL1/2 RNAs could not silence V. dahliae DCL genes. Thus, these Bc-DCL RNA regions chosen from outside the conserved regions for RNAi are very specific to B. cinerea DCL genes and do not cause off-target effects. Thus, specific target region selection could circumvent potential off-target problem. This RNAi-based disease management strategy targeting specific regions of pathogen DCL genes is efficient and safe in controlling multiple fungal pathogens that use sRNA effectors.

**Discussion**

We previously discovered that B. cinerea delivered sRNA effectors into plant cells to induce cross-kingdom RNAi of host plant immunity genes. Here, we show that another plant fungal pathogen V. dahliae also likely uses sRNAs as effectors and these sRNAs are loaded into Arabidopsis AGO1 for silencing of host genes. A similar virulence mechanism was recently observed in animal systems as well. For example, animal parasites, such as H. polygyrus, deliver miRNAs to mammalian cells and silence host genes involved in innate immunity. Thus, cross-kingdom RNAi has evolved in pathogens and pests of both plant and animal systems as a conserved virulence mechanism. Furthermore, we demonstrate that sRNAs generated from the host plant cells are also transferred into fungal B. cinerea cells, which provided the first example of bidirectional cross-kingdom RNAi and sRNA trafficking between a plant host and a fungal pathogen. Plant hosts expressing Bc-DCL-targeting sRNAs can effectively control grey mould disease.

In this study, we provide solid evidence to demonstrate that environmental RNAi also exists in fungi. We show that fungal pathogen B. cinerea is capable of taking up external sRNAs and long dsRNAs. In C. elegans, RNA uptake from the environment requires dsRNAs that are longer than 50 bp; shorter dsRNAs or mature sRNAs cannot be effectively taken up by C. elegans. Some herbivorous insects are also capable of taking up sRNAs that are longer than 50–60 bp, but not sRNAs. Here, we show that B. cinerea can take up both sRNAs and dsRNAs directly, and both can induce silencing of B. cinerea genes, suggesting that the RNA uptake pathways, or pathways that may differ among organisms. Consistent with that, the Systemic RNAi-deficient (SID) genes that are important for RNA uptake and transport37–40,49,50 are mostly C. elegans specific and not present in plants, fungi, oomycete or even other animals50. Future studies are needed to identify the sRNA and dsRNA trafficking pathways between pathogens and hosts and to elucidate the mechanisms of RNA uptake in fungi.

Eukaryotic pathogens, including fungi and oomycetes, cause billions of dollars in crop loss annually. Currently, fungicides and chemical spraying are still the most common disease control strategy, yet they pose serious threats to human health and environments. In the last few years, the stable plant transformation-based HIGS system has been proven to efficiently control certain pests, nematodes, filamentous pathogens and parasitic plants in various plant models and crop species. However, these successful HIGS studies relied on a plant transformation system that is not available for many crops. Our discovery of RNA uptake by B. cinerea makes it possible to directly use such external dsRNAs and sRNAs for disease management. We show that applying Bc-DCL1/2-sRNAs and –dsRNAs on the surface of various fruits, vegetables and flowers can efficiently control grey mould diseases. This RNAi-based new generation of ‘RNA fungicides’ could circumvent the technical limitation of transformation and the public’s concerns about GMOs and provide an easy-to-use and environmentally friendly disease management tool for crop production and storage. Furthermore, such RNA-based strategies could be easily designed to target multiple pathogens.

**Methods**

**Plasmid construction.** The plasmids pHellegate–Bc-DCL1/2 and pHellgate–Vd-DCL1/2 were constructed following the methods outlined for the Helligate8.0 system31. The Bc-DCL1/2 RNAi fragment was obtained by integrating Bc-DCL1 (252 bp) and Bc-DCL2 (238 bp) by overlapping PCR. The Bc + Vd-DCLs RNAi fragment was obtained by integrating the 315 bp Bc-DCL1/2 RNAi fragment (164 bp of Bc-DCL1 and 151 bp of Bc-DCL2) and RNAi fragments of Vd-DCL1 (156 bp) and Vd-DCL2 (156 bp) via overlapping PCR. The RNAi fragments were cloned separately into pDONR207 using Gateway BP clonase (Life Technologies), then into the destination vector pHellgate8.0 using Gateway LR clonase (Life Technologies). For the pTRV2–Bc-DCL1/2 plasmid, the pDONR207–Bc-DCL1/2 vector was used to carry out the LR reaction with pTRV2–EV to obtain pTRV2–Bc-DCL1/2 using LR clonase (Life Technologies, Carlsbad, CA).

**In vitro synthesis of dsRNAs and sRNAs.** Following the MEGAscript RNAi Kit instructions (Life Technologies), the T7 promoter sequence was introduced into both the 5′ and the 3′ ends of the RNAi fragments by PCR. After purification, the DNA fragments containing T7 promoters at both ends were used for in vitro transcription. To obtain sRNAs, the synthesized dsRNAs were digested with ShortCut RNase III (NEB), and sRNAs were subsequently cleaned using the mirVana miRNA Isolation Kit (Life Technologies).

**In vitro RNA fluorescence labelling for confocal microscopy.** Bc-DCL1/2–dsRNAs were labelled using the Fluorescein RNA Labelling Mix Kit following the manufacturer’s instructions (Sigma). The fluorescent Bc-DCL1/2–sRNAs were obtained from the digestion of fluorescent Bc-DCL1/2–dsRNAs with the ShortCut RNase III enzyme (NEB). For confocal microscopy examination of fluorescent RNA trafficking into fungal mycelium, 4 µl of 10 ng µl−1 fluorescent RNAs were applied to 4 µl of 10^4 spores ml−1 glistening spores and grown on ME agar medium that had been prepared on microscopic slides. After 12 h, the mycelium was treated with KCl buffer or 75 U Micrococcal Nuclease enzyme (Thermo Scientific) at 37 °C for 30 min. The fluorescent signal was analysed using a Leica SP5 confocal microscope.

To detect fluorescent RNA trafficking into B. cinerea protoplasts, 15 µl of 100 ng µl−1 fluorescent RNAs was added into 10^5 B. cinerea spores cultured in 5 ml of liquid yeast extract peptone dextrose (YEPD). After 20 h, the protoplasts were isolated using lysing enzyme from Trichoderma harzianum (Sigma), followed by treatment with KCl buffer or 75 U of Micrococcal Nuclease enzyme (Thermo Scientific) at 37 °C for 30 min. Fluorescence was examined using a Leica SP5 confocal microscope.

To autocorrect artificial fluorescence and/or auto-fluorescence of fungal mycelium and protoplasts, the confocal microscope settings were adjusted using the water-treated control samples (mycelium or protoplast), and the same settings (except for the focus) were applied to all the samples.

**Isolation of B. cinerea protoplasts from infected Arabidopsis plants.** Arabidopsis plants were sprayed with B. cinerea del1 del2 mutant strain or inoculation medium (Mock) for 48 h, and then the leaf tissues were subjected to sequential protoplast preparation based on the different compositions of cell walls between fungus and plant. The same amount of B. cinerea del1 del2 spores that used for infection were cultured on YEPD liquid medium for two days. All the mycelia collected from the culture were mixed with the mock-treated plant samples and used as a control of the fungal protoplast isolation procedure to ensure the purity of the fungal protoplasts. First, plant protoplast extraction from the leaf tissues was performed as described previously22. The plant protoplasts were sheared using a 1% Triton X-100 (Sigma) treatment, and were washed five times to remove plant contents. Then the fungal protoplasts were isolated using lysing enzyme from Trichoderma harzianum (Sigma) to obtain pure fungal protoplasts.

**Preparation of N. benthamiana total RNA carrying Bc-DCL1/2–sRNAs and –dsRNAs, as well as YFP–sRNAs and –dsRNAs.** Four-week-old N. benthamiana plants were infiltrated with an A. tumefaciens strain carrying pHellgate–EV, pHellegate–Bc-DCL1/2 or pHellgate–YFP. The leaf tissue was harvested 2 dpi and used for total RNA extraction.

**External application of RNAs on the surface of plant materials.** All RNAs were adjusted to a concentration of 20 ng µl−1 with RNase-free water before use. For the concentration series, RNAs were diluted to 10, 5 and 1 ng µl−1. For in vitro synthetic YFP–sRNAs or YFP–dsRNAs, and Bc-DCL1/2–sRNAs and –dsRNAs, 20 µl of RNA (20 ng µl−1) was dropped onto the surface of each plant specimen. Then the B. cinerea inoculum was applied on the same spot. For N. benthamiana total RNA extracts, which is easy and cheap to obtain large quantity, the surface of each plant specimen was evenly sprayed with approximately 400 µl of 20 ng µl−1 RNAs.
References

1. Ghidhibal, M. & Zamore, P. D. Small silencing RNAs an expanding universe. Nat. Rev. Genet. 10, 94–108 (2009).

2. Baulcombe, D. RNA silencing in plants. Nature 431, 356–363 (2004).

3. Vaucheret, H. Plant ARGOAUTs. Trends Plant Sci. 13, 350–358 (2008).

4. Hujanen, G. & Simard, M. J. Argonauta protein: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. 9, 22–32 (2008).

5. Weiberg, A. & Jin, H. L. Small RNAs: the secret agents in the plant-pathogen interactions. Curr. Opin. Plant Biol. 26, 87–94 (2015).

6. Weiberg, A., Wang, M., Bellinger, M. & Jin, H. Small RNAs: a new paradigm in plant-microbe interactions. Annu. Rev. Physiopathol. 52, 495–516 (2014).

7. Buck, A. H. et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat. Commun. 5, 5488 (2014).

8. Cheng, G. F., Luo, R., Hu, C., Cao, J. & Jin, Y. X. Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with Schistosoma japonicum. Parasitology 140, 1751–1761 (2013).

9. Garcia-Silva, M. R. et al. Extracellular vesicles shed by Trypanosoma cruzi are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. Parasitol. Res. 113, 285–304 (2014).

10. Zamanian, M. et al. Release of small RNA-containing exosome-like vesicles from the human filamentous fungus Branchiostoma lagunai. PLoS Negl. Trop. Dis. 9, e004009 (2015).

11. Quntamia, J. F. et al. Extracellular Oncorhynchus-derived small RNAs in host nodules and blood. Parasite Vectors 8, 58 (2015).

12. Baum, J. et al. Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. 25, 1322–1326 (2007).

13. Mao, Y. R. et al. Silencing a cotton bollworm P450 monoxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat. Biotechnol. 25, 1307–1313 (2007).

14. Liu, J., Todd, T. C., Oakley, T. R., Lee, J. & Trick, H. N. Host-derived suppression of nematode reproductive and fitness genes decreases fecundity of Heterodera glycines Ichinose. Plant Cell 232, 775–785 (2010).

15. Nowara, D. et al. HIGS: Host-Induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. Plant Cell 21, 3310–3411 (2010).

16. Koch, A. et al. Host-induced gene silencing of the obligate biotrophic fungal pathogen Blumeria graminis. Plant Cell 21, 3310–3411 (2010).

17. Johann, S. N. et al. Plant-mediated gene silencing restricts growth of the potato late blight pathogen Phytophthora infestans. J. Exp. Bot. 66, 2785–2794 (2015).

18. Vega-Arreguin, J. C., Jalloh, A., Bó, J. & Moffett, P. Recognition of an Avr3a homologue plays a major role in mediating nonhost resistance to Phytophthora capsici in Nicotiana species. Mol. Plant Microbe Interact. 27, 770–780 (2014).

19. Nunez, C. & Dean, R. A. Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. Mol. Plant Pathol. 13, 519–529 (2012).

20. Williamson, B., Tuzynska, B., Tuzynski, P. & van Kan, J. A. L. Botrytis cinerea: Physiological and molecular aspects of the cause of grey mould disease. Mol. Plant Pathol. 15, 1313–1345 (2015).

21. Sanjuán, S. et al. Host-mediated gene silencing of a single effector gene from the potato pathogen Phytophthora infestans imparts partial resistance to late blight disease. Funct. Integr. Genomics 15, 697–706 (2015).

22. Humbert, H. et al. Diversity of pathways generating microRNA-like RNAs and dicer-independent small interfering RNAs in fungi. Mol. Cell 38, 803–814 (2010).

23. Jin, H. L. & Zhu, J. K. How many ways are there to generate small RNAs? Mol. Cell 38, 777–777 (2010).

24. Hellwell, C. A. & Waterhouse, P. M. Constructs and methods for hairpin RNA-mediated gene silencing in plants. Methods Enzymol. 392, 34–35 (2005).

25. Liu, Y. L., Schiff, M. & Dinesh-Kifer, S. P. Virus-induced gene silencing in tomato. Plant J. 31, 777–786 (2002).

26. Song, J. Q. et al. Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. Proc. Natl Acad. Sci. USA 100, 9128–9133 (2003).

27. Feinberg, E. H. & Hunter, C. P. Caenorhabditis elegans Sd-2 is required for environmental RNA interference. Proc. Natl Acad. Sci. USA 104, 10565–10570 (2007).

28. Whangbo, J. S. & Hunter, C. P. Environmental RNA interference. Trends Genet. 24, 297–305 (2008).

29. McWan, D. L., Weisman, A. S. & Hunter, C. P. Uptake of extracellular double-stranded RNA by S2-2. Mol. Cell 47, 746–754 (2012).

30. Feinberg, E. H. & Hunter, C. P. Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301, 1545–1547 (2003).

31. Yovelta, A. et al. Environmental RNAi in herbivorous insects. RNA 21, 840–850 (2015).

32. San Miguel, K. & Scott, J. G. The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. Pest Manage. Sci. 72, 801–809 (2016).

33. Baulcombe, D. C. VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. Curr. Opin. Plant Biol. 26, 141–146 (2015).

34. Govindarajulu, M., Epstein, L., Wroblewski, T. & Michelmore, R. W. Host-induced gene silencing inhibits the biotrophic pathogen causing downy mildew of lettuce. Plant Biotechnol. J. 13, 875–883 (2015).

35. Sales, M. C. et al. The endosymbiotic pathway mediates cell entry of dsRNA to induce RNA interference. Nat. Cell Biol. 8, 793–802 (2006).

36. Bolognesi, R. et al. Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (Diabrotica virgifera virgifera LeConte). PLoS ONE 7, e47534 (2012).

37. Ellendorff, V., Fradin, E. F., de Jonge, R. & Thomma, B. P. J. H. RNA silencing is required for Arabidopsis defence against Verticillium wilt disease. J. Exp. Bot. 60, 591–602 (2009).

38. Zhang, X. et al. Arabidopsis argonaute 2 regulates innate immunity via miRNA393(*)-mediated silencing of a Golgi-localized SNARE gene, MEMB12. Mol. Cell 42, 356–366 (2011).

39. Hinas, A., Hunter, A. J., Hunter, C. P. SID-5 is an endosymbiotic-associated protein required for efficient systemic RNAi in C. elegans. Curr. Biol. 22, 1938–1943 (2012).

40. Wang, M., Weiberg, A. & Jin, H. Pathogen small RNAs: a new class of effectors for pathogen attacks. Mol. Plant Pathol. 16, 219–223 (2015).

41. Hellwell, C. A. & Waterhouse, P. Constructs and methods for high-throughput gene silencing in plants. Methods 30, 289–295 (2003).

42. Yoo, S. D., Cho, Y. H. & Sheen, J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572 (2007).

Acknowledgements

We thank H. Vaucheret for the agol-27 seeds, M. Coffey for the V. dahliae JR2 strain, I. Kaloshin for providing the growth room space for VIGS experiments, and Y. Li for editing the paper. This work was supported by grants from National Institute of Health (GM069308), National Science Foundation (IOS-1257576, IOS-1557981) and an AES-CE Award (PFA-751797) awarded to H.J.

Author contributions

H.J. conceived the idea. M.W. and H.J. designed the experiments. M.W. performed most of the experiments and analysed data. A.W. profiled the sRNAs from del1 del2 and WT strains and analysed the data. F.M.L. and H.D.H. conducted bioinformatics analysis on sRNA libraries. B.T. provided Vd genome sequence for JR2 strain. M.W., A.W. and H.J. wrote the manuscript.

Additional information

Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.J.

Competing interests

The authors declare no competing financial interests.