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

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Abstract

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 *B. 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 gray mold disease. Such pathogen gene-targeting RNAs represent a new generation of environmentally-friendly fungicides.

Small RNAs (sRNAs) are short non-coding regulatory RNAs that are mostly generated by Dicer or Dicer-like (DCL) endoribonucleases from double-stranded RNAs (dsRNAs) or single-stranded RNAs (ssRNAs) with hairpin structures[1,2]. Mature sRNAs are loaded into Argonaute (AGO) proteins and induce silencing of genes with fully or partially complementary sequences[3,4]. This phenomenon is called RNA interference (RNAi) and is widely conserved in most eukaryotic organisms. We have recently discovered that *Botrytis*

cinerea, an aggressive plant fungal pathogen, delivers small RNAs (Bc-sRNAs) into host plant cells and utilizes host RNAi machinery to suppress host immunity genes[5]. These pathogen sRNAs mediate cross-kingdom RNAi in plant hosts and represent a novel class of pathogen effectors that inhibit host immunity for successful infection[6,7]. Similarly, some animal parasites, such as gastrointestinal nematode *Heligmosomoides polygyrus,* also deliver sRNAs into mammalian cells and target host genes involved in innate immunity[8-12]. 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 insects[13,14], nematodes[15,16], fungi[17,18], and oomycetes[19,20], 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 caused 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 gray mold disease[22,23]. *Verticillium dahliae* is another economically important fungal pathogen, which causes wilt disease on a wide range of plant species[24,25]. 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 graminis*[17,26,27], *Puccinia tritici*[28], *Fusarium* spp.[18,29,30], *Phytophthora infestans*[19,31], and *Phytophthora capsici*[20], but it is restricted to plants with established transformation methods, which is 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 gray mold 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 *DCL*s were knocked out[5]. 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), as well as on cultured media (Supplementary Fig. 1a). 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 retrotransposons mostly long terminal repeats (LTRs) (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 antisense 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[5]. 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 gray mold 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[34] 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% to 4.8% sequence homology to *Arabidopsis DCL*s (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 when compared with 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 to 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 gray mold disease in tomato plants, we introduced the same *Bc-DCL1/2* fragment into the

tobacco rattle virus (TRV) silencing system[35], 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 an potato late blight resistance gene *RB* that is not present in tomato[36] 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 *DCL*s 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. Gray mold 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, while the majority of crop plants do not have available genetic manipulation tools. Moreover, genetically modified organisms (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*[37-40] and certain insects[41,42]. Spraying dsRNAs targeting the *actin* gene of potato beetle *Leptinotarsa decemlineata* on potato leaves inhibited larval growth[42]. *In vitro* treatment of fungal pathogen *F. graminearum* with dsRNAs targeting *CYP51* genes also inhibited fungal growth[18], but there is no direct evidence demonstrating environmental dsRNAs move into fungal cells to silencing 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-labeled *Bc-DCL1/2*-dsRNAs by *in vitro* transcription using Fluorescein-12-UTP, and we generated Fluorescein-labeled *Bc-DCL*1/2-sRNAs by RNase III *in vitro* processing of the Fluorescein-labeled 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-labeled *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 liquid

culture of *B. cinerea* geminating 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 labeled 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-DCL*1/2-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[14,17,18,43,44], none have 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[40,45,46]. Transgenic *Arabidopsis* expressing hairpin *Bc-DCL*1/2-RNAs, which showed a high level of processed 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 hpi, 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 prior to protoplast formation was used as a control 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 translocated into *B. cinerea*. The Bc-DCL-independent sRNA *Bc*-siR1498 that we previously identified[5] 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, 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-DCL*1/2 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 in pure water) were externally applied on fruits (tomato, strawberry, and grape), vegetables (lettuce and onion), flower petals (rose), and *Arabidopsis* leaves, and followed with *B. cinerea* infection. Water, *YFP*-dsRNAs and -sRNAs were used as controls. All the plants treated with *Bc-DCL1/2*-sRNAs and -dsRNAs developed much weaker disease symptoms (Fig. 4b,c and Supplementary Fig. 4a) and supported significantly less growth of *B. cinerea* (Fig. 4c and Supplementary Fig. 4a) compared to the controls treated with water, *YFP*-dsRNAs or

YFP-sRNAs. Furthermore, the expression of *Bc-DCL1* and *Bc-DCL2* was suppressed by the treatment of *Bc-DCL1/2*-dsRNAs and -sRNAs in the *B. cinerea* isolated from the infection sites (Supplementary Fig. 4b), suggesting that externally applied *Bc-DCL1/2*-sRNAs and -dsRNAs can inhibit pathogen virulence, probably via the suppression of *Bc*-sRNA effector biogenesis and fungal growth. We also tested the efficiency of this plant protection using various concentrations of *Bc-DCL1/2*-sRNAs and -dsRNAs, and our data indicated that such RNAs remained functional at a concentration as low as 5 ng/μl, but not at 1 ng/μl (Supplementary Fig. 5), although the higher concentration of RNAs seems to provide stronger protection (Fig. 4b,c and Supplementary Fig. 5). We also examined the duration of plant protection against gray mold 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 post 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, while we only used RNAs dissolved in water. Mixing the RNAs with reagents that stabilize RNAs could likely 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. In order 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 empty vector (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 and 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 to 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. dahilae* that displayed reduced virulence on *Arabidopsis ago1-27* mutant[47], just like *B. cinerea*. *V. dahilae* 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. dahilae* also utilizes cross-kingdom RNAi to suppress host immunity genes by hijacking host AGO1 for its virulence, and whether *V. dahilae* sRNAs are indeed loaded into host AGO1 protein during infection, we performed RNA

immunoprecipitation (RIP) assay on *V. dahilae*-infected *Arabidopsis* roots using *Arabidopsis* AGO1- and AGO2-antibodies as described previously[5,48]. *Arabidopsis ago1-27* mutant is less susceptible to *V. dahilae* than WT plants in both soil and root culture conditions (Fig. 6a,b). The *ago2-1* mutant shows no difference compared to WT plants, suggesting that At-AGO2 is not involved in the gene regulation during plant - *V. dahilae* 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[5], we found that 99 At-AGO1-associated *Vd*-sRNAs have *Arabidopsis* target genes, whereas only 13 At-AGO2-associated *Vd*-sRNAs have predicted *Arabidopsis* targets (Supplementary Table 2,3). These results suggest that *V. dahilae* 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. dahilae* and *B. cinerea* to test whether the RNAi-based method can simultaneously control both fungal diseases. *V. dahilae* 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. dahilae* 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% to 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. dahilae* (Fig. 6d-g). To determine whether the silencing effect is indeed gene-specific, we infected *Bc-DCL1/2*-RNAi plants with *V. dahilae.* These plants only express *Bc-DCL1/2*-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 silencing *V. dahilae DCL* genes. Thus, these *Bc-DCL* DNA 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[5]. Here, we show that another plant fungal pathogen *V. dahilae* also 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[8]. 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 gray mold disease.

In this study, we provide solid evidences 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*[37-40,49]. Some herbivorous insects are also capable of taking up dsRNAs that are longer than 50–60 bp, but not sRNAs[41,46]. 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 channels or pathways may differ among organisms. Consistent with that, the *Systemic RNAi-deficient* (*SID*) genes that are important for RNA uptake and transport[37-40,4950] are mostly *C. elegans*-specific and not present in plants, fungi, oomycete, or even other animals[50]. 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, fungicide and chemical spray is still the most common disease control strategy, yet they pose serious threats to human health and environments. Over the last few years, the stable plant transformation-based HIGS system was proven to efficiently control certain pests, nematodes, filamentous pathogens, and parasitic plants in various plant models and crop species[13-15,17,18,21]. 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 gray mold 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.

Materials and Methods

Plasmid construction

The plasmids pHellsgate-*Bc-DCL1/2* and pHellsgate-*Bc+Vd-DCLs* were constructed following the methods outlined for the Hellsgate8.0 system[51]. The *Bc-DCL1/2* RNAi fragment was obtained by integrating *Bc-DCL1* (252 bp) and *Bc-DCL2* (238 bp) via 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, Carlsbad, CA), then into the destination vector pHellsgate8.0 using Gateway®

LR clonase (Life Technologies, Carlsbad, CA). For the pTRV2-*Bc-DCL1/2* plasmid, the pDONR207-*Bc-DCL1/2* vector was used to do LR reaction with pTRV2-EV to obtain pTRV2-*Bc-DCL1/2* using LR clonase (Life Technologies, Carlsbad, CA)[5].

In vitro synthesis of dsRNAs and sRNAs

Following the MEGAscript® RNAi Kit instructions (Life Technologies, Carlsbad, CA), T7 promoter sequence was introduced into both 5' and 3' ends of the RNAi fragments by PCR, respectively. 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, Ipswich, MA), and sRNAs were subsequently cleaned using the *mir*Vana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA).

In vitro RNA fluorescence labeling for confocal microscopy

Bc-DCL1/2-dsRNAs were labeled using the Fluorescein RNA Labeling Mix Kit following the manufacture's instructions (Sigma, St. Louis, MO). The fluorescent *Bc-DCL1/2*-sRNAs were obtained from the digestion of fluorescent *Bc-DCL1/2*-dsRNAs with the ShortCut® RNase III enzyme (NEB Ipswich, MA). For confocal microscopy examination of fluorescent RNA trafficking into fungal mycelium, 4 μl of 100ng/μl fluorescent RNAs were applied onto 4 μl of 10^5 spores/ml geminating spores and grew on ME agar medium that prepared on microscopic slides. After 12 hours, the mycelium were treated by KCl buffer or 75 U Micrococcal Nuclease enzyme (Thermo Scientific, Waltham, MA) at 37°C for 30 minutes. The fluorescent signal was analyzed using a Leica SP5 confocal microscope.

To detect fluorescent RNA trafficking into *B. cinerea* protoplasts, 15 μl of 100 ng/μl fluorescent RNAs were added into 10^7 *B. cinerea* spores cultured in 5 ml liquid yeast extract peptone dextrose (YEPD). After 20 h, the protoplasts were isolated using lysing enzyme from *Trichoderma harzianum* (Sigma, St. Louis, MO), followed by treatment with KCl buffer or 75 U Micrococcal Nuclease enzyme (Thermo scientific, Waltham, MA) 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 spray-infected with *B. cinerea dcl1 dcl2* mutant strain as well as inoculation medium for 48 hours, and then the leaf tissues were subjected to sequential protoplast preparation based on the different compositions of cell walls between fungus and plant. First, plant protoplast extraction from the leaf tissues was performed as described previously[52]. The plant protoplasts were sheared using a 1% Triton X-100 (Sigma, St. Louis, MO) treatment, and were washed 5 times to remove plant contents. Then the fungal protoplasts were isolated using lysing enzyme from *Trichoderma harzianum* (Sigma, St. Louis, MO) 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 *A. tumefaciens* strain carrying pHellsgate-EV, pHellsgate-*Bc-DCL1/2* or pHellsgate-*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 with RNase-free water before use. For the concentration series, RNAs were diluted to 10, 5, and 1 ng/µl. For *in vitro* synthetic *YFP*-sRNA and -dsRNAs, and *Bc-DCL1/2*-sRNAs and -dsRNA, 20 µl RNA (20 ng/µl) 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 400µl of 20 ng/µl RNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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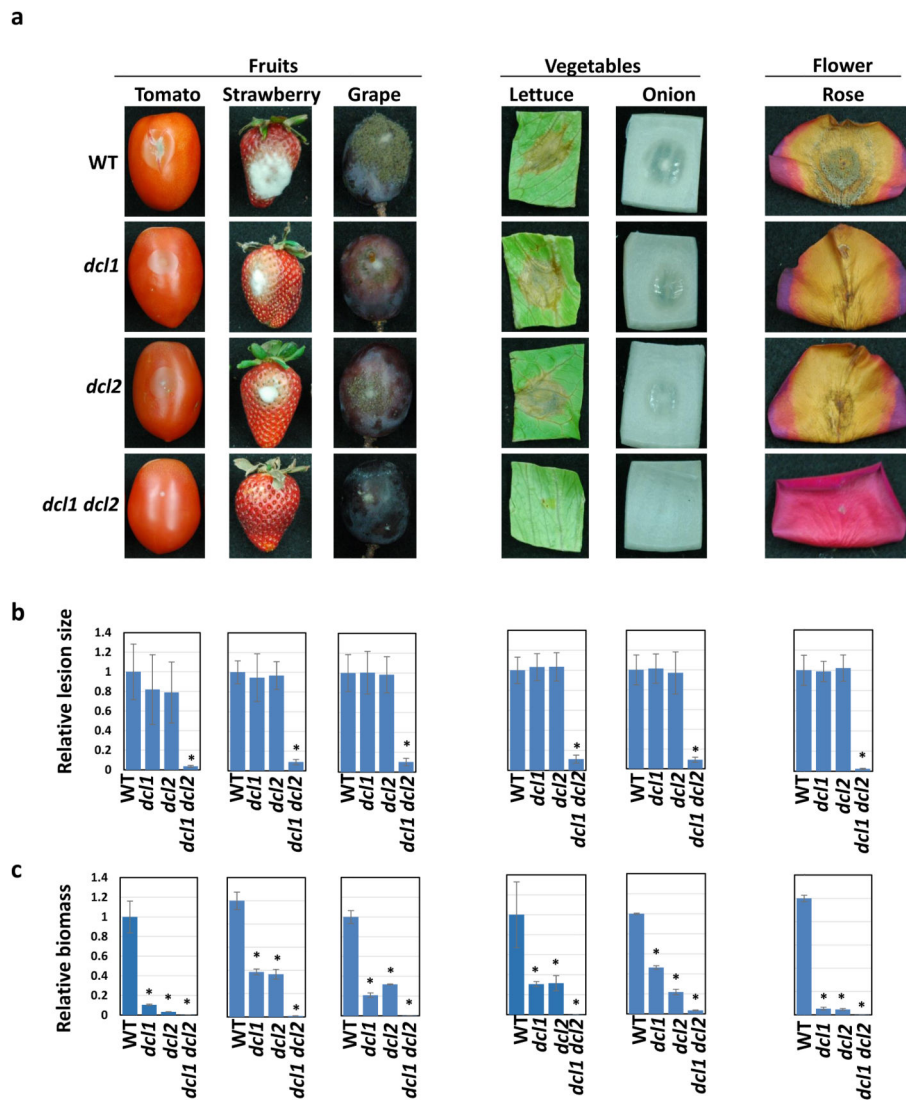
Figure 1. B. cinerea dcl1 dcl2 double mutant, but not the dcl1 or dcl2 single mutant, displays reduced virulence on fruits, vegetables, and flower petals

(a) *B. cinerea dcl1 dcl2* double mutant shows compromised virulence on fruits (tomato, strawberry, and grape), vegetables (lettuce and onion), and flower petals (rose), while *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, and error bars indicate the standard deviations (SD) of 10 samples. (c) *B. cinerea* relative DNA content (relative biomass) was measured by quantitative PCR. Error bars indicate the SD of three technical replicates. Asterisks indicate statistically significant differences (P < 0.01). Similar results were obtained from at least three biological replicates.
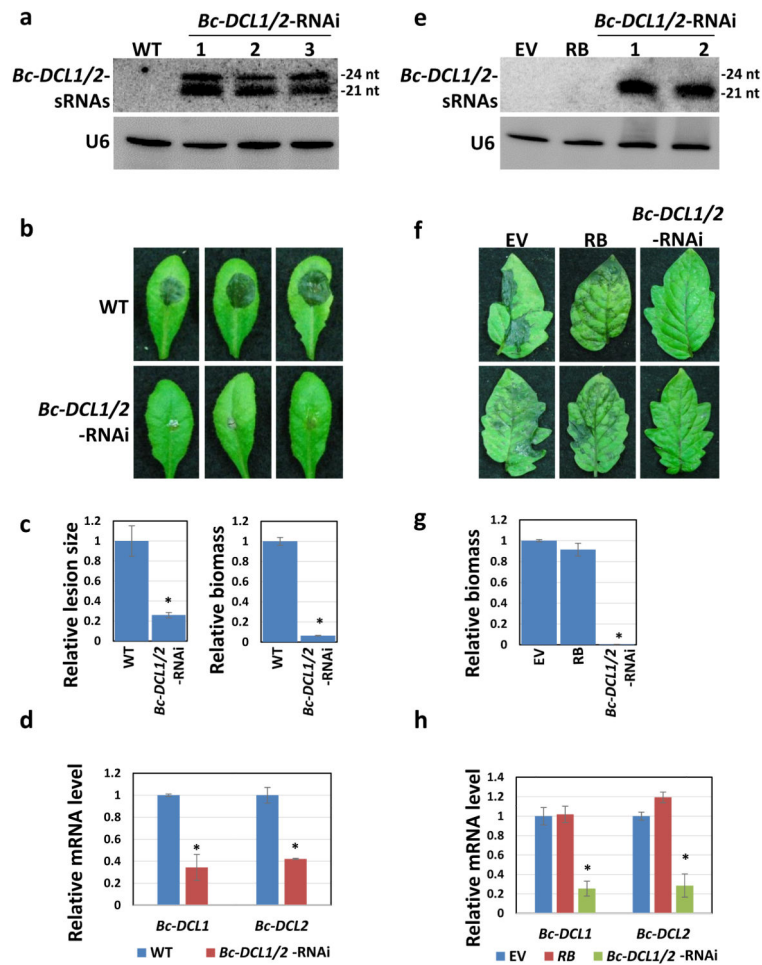
Figure 2. *Arabidopsis* **and tomato** *Bc-DCL1/2***-RNAi plants confer enhanced resistance against** *B. cinerea* **infection**

(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*. Similar results were obtained from three biological replicates. (c) Relative lesion sizes were measured 3 dpi using imageJ, and error bars indicate the SD of 10 samples. *B. cinerea* relative biomass was measured 3 dpi by quantitative PCR, and error bars indicate the SD 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* compared to control plants (EV and RB). Similar results were obtained from three biological replicates. (g) *B. cinerea* relative biomass was measured 3 dpi, and error bars indicate the SD 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 ($P < 0.01$).
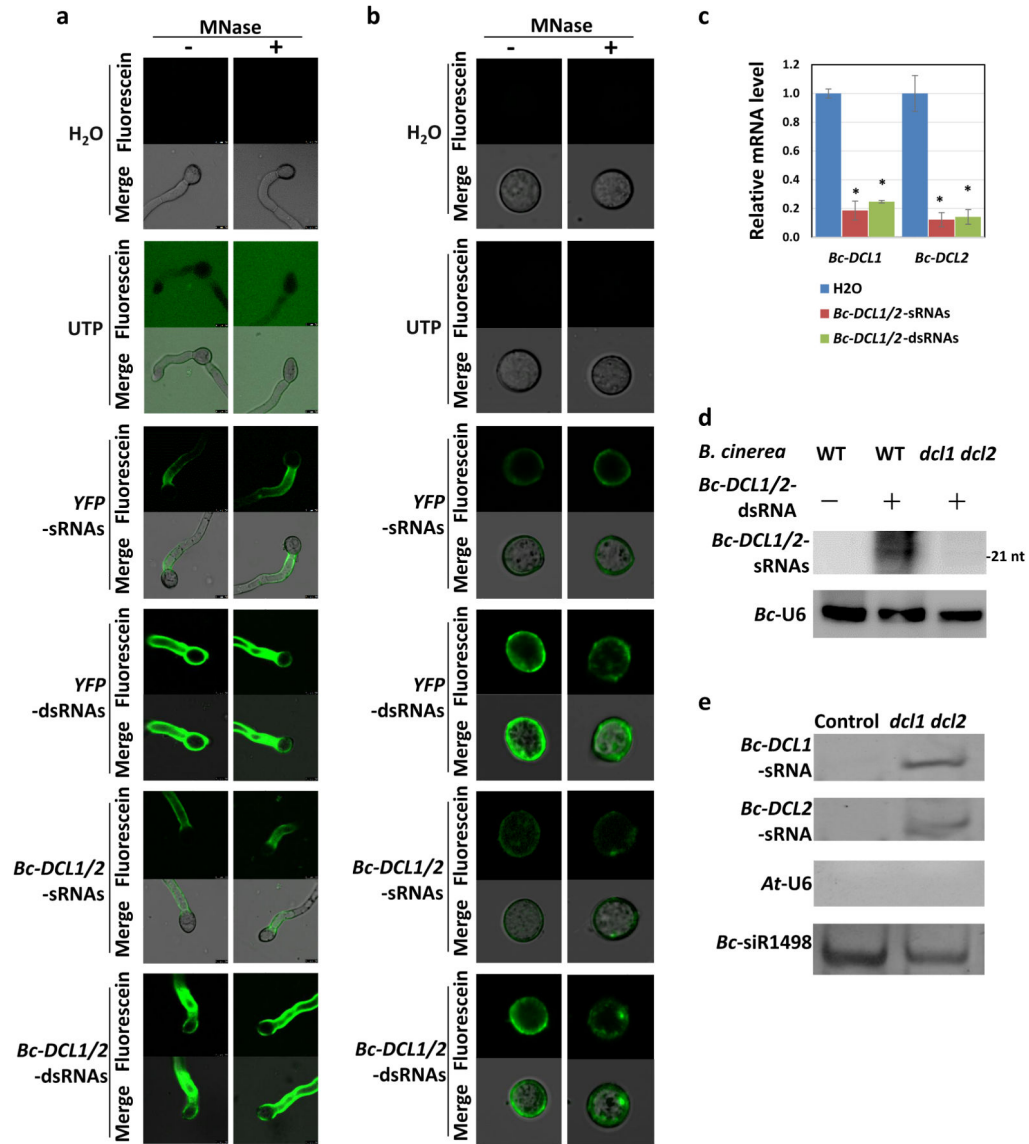
Figure 3. Environmental *Bc-DCL1/2*-sRNAs and -dsRNAs are taken into *B. cinerea* cells and where they silence fungal *DCL* genes; *Bc-DCL1/2*-sRNAs move from plants into fungal cells
(a) Fluorescein-labeled *Bc-DCL1/2*-sRNAs and -dsRNAs, as well as *YFP*-sRNAs and -dsRNAs, were applied onto *B. cinerea* spores and fluorescent signals were detected in the *B. cinerea* cells at 12 h post culturing on agar (malt extract) ME medium. Fluorescence signals remained visible in the *B. cinerea* cells after MNase treatment. Fluorescein-UTP and water were used as controls. (b) Fluorescein-labeled *Bc-DCL1/2*-sRNAs and -dsRNAs, as well as *YFP*-sRNAs and -dsRNAs, were observed in *B. cinerea* protoplasts after MNase treatment. Fluorescent sRNAs or dsRNAs were applied onto germinated *B. cinerea* spores and protoplasts were isolated after culturing for 20 h. The fluorescent signals were detected within fungal protoplasts, and the MNase enzyme treatment did not reduce the fluorescent signal intensities. (c) *Bc-DCL1* and *Bc-DCL2* were down-regulated in *Bc-DCL1/2*-RNA-treated *B. cinerea*. (d) *In vitro* synthesized *Bc-DCL1/2*-dsRNAs were taken up and processed into sRNAs after co-culturing with the *B cinerea* WT strain but not with the *dcl1*

dcl2 mutant. (e) *Bc-DCL1*-sRNAs and *Bc-DCL2*-sRNAs were detected by stem-loop RT-PCR in *B. cinerea dcl1 dcl2* protoplasts after infection on *Arabidopsis Bc-DCL1/2*-RNAi plants but not in mock-treated *Arabidopsis Bc-DCL1/2*-RNAi plants mixed with *B. cinerea dcl1 dcl2* mycelium prior to protoplast formation. Similar results were obtained from two biological replicates.
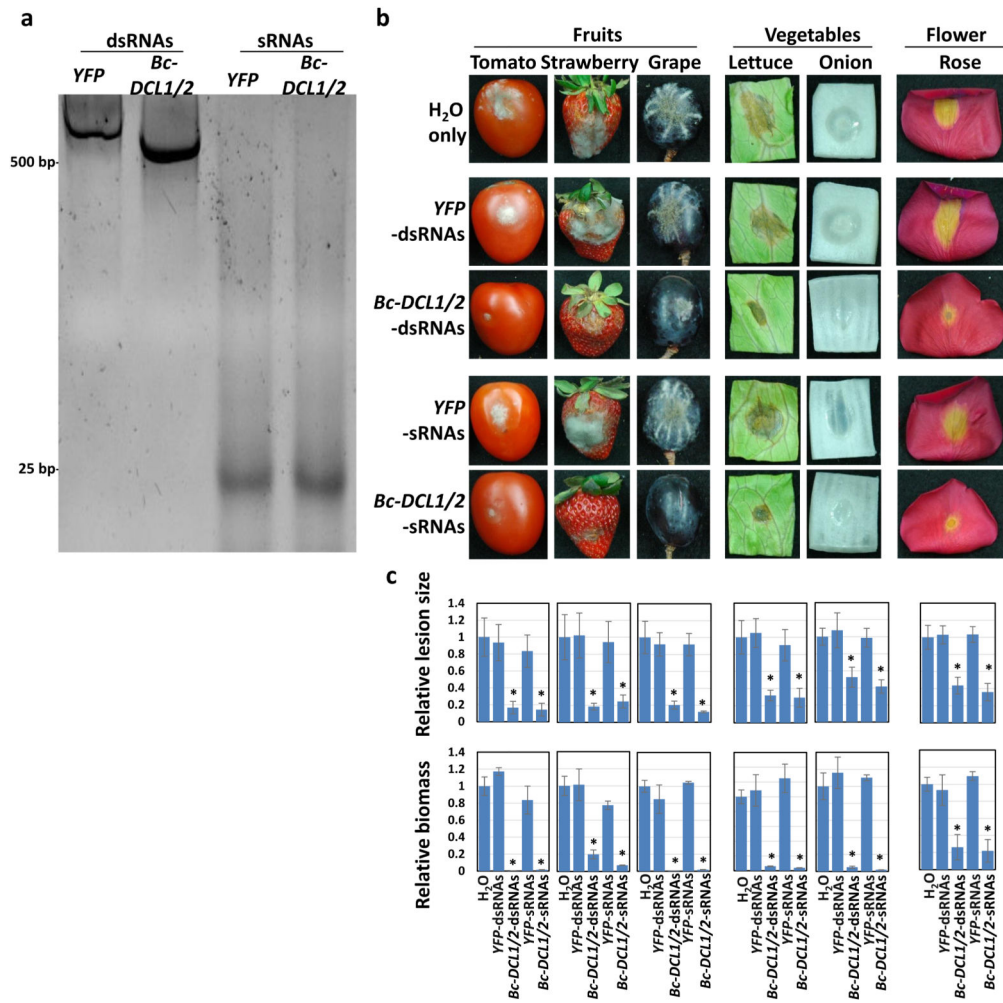
Figure 4. Externally applied *Bc-DCL1/2*-sRNAs and -dsRNAs inhibited pathogen virulence on fruits, vegetables, and flower petals

(a) *Bc-DCL1/2*-dsRNAs and -sRNAs, as well as *YFP*-dsRNAs and -sRNAs, were synthesized and processed, and 100 ng of RNAs was analyzed on a native PAGE gel to check the quality. (b) External application of *Bc-DCL1/2*-dsRNAs and -sRNAs (20ul of 20 ng/μl synthetic RNAs) inhibits the virulence of *B. cinerea* on fruits (tomato, strawberry, and grape), vegetables (lettuce and onion), and flower petals (rose), compared to the treatments using water, *YFP*-dsRNAs and -sRNAs. (c) The relative lesion sizes and fungal biomass were measured 3 dpi for lettuce, onion, rose, and strawberry and 5 dpi for tomato and grape fruits using ImageJ software and quantitative PCR, respectively. Error bars indicate the SD of 10 samples and three technical repeats for the relative lesion sizes and relative biomass, respectively. Asterisks indicate statistically significant differences ($P < 0.01$). Similar results were obtained from three biological replicates.
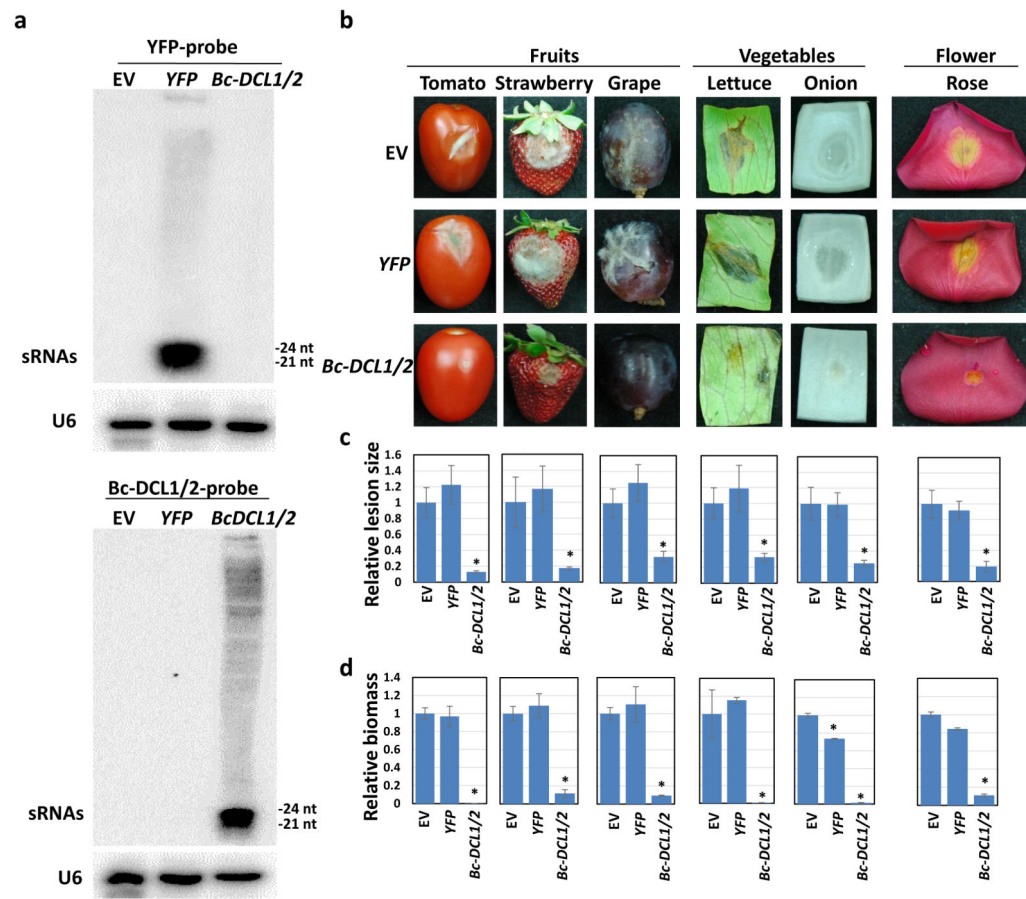
Figure 5. Treatment with *N. benthamiana* RNA extracts containing *Bc-DCL1/2*-sRNAs and -dsRNAs reduces gray mold disease symptoms caused by *B. cinerea*

(a) Total RNA extracted from the *N. benthamiana* plants expressing Hellsgate empty vector (EV), *YFP* or *Bc-DCL1/2* RNAi constructs was examined by Northern blot analysis to measure the expression levels of *YFP*- and *Bc-DCL1/2* sRNAs. (b) *N. Benthamiana* total RNAs containing *Bc-DCL1/2*-RNAs (20 ng/μl) were sprayed onto fruits (tomato, strawberry, and grape), vegetables (lettuce and onion), and flower petals (rose) reduced grey mold disease symptoms, when compared with the application of *N. Benthamiana* total RNA extracts from plants expressing *YFP*-sRNAs and -dsRNAs or no sRNAs (EV). (c) The relative lesion sizes were measured 3 dpi for lettuce, onion, rose and strawberry, and 5 dpi for tomato and grape fruits using imageJ, and error bars represent the SD of 10 plant samples. The relative biomass of *B. cinerea* was also calculated with quantitative PCR, and error bars represent the SD of three technical replicates. Asterisks indicate statistically significant differences (P < 0.01). Similar results were observed from three biological replicates.
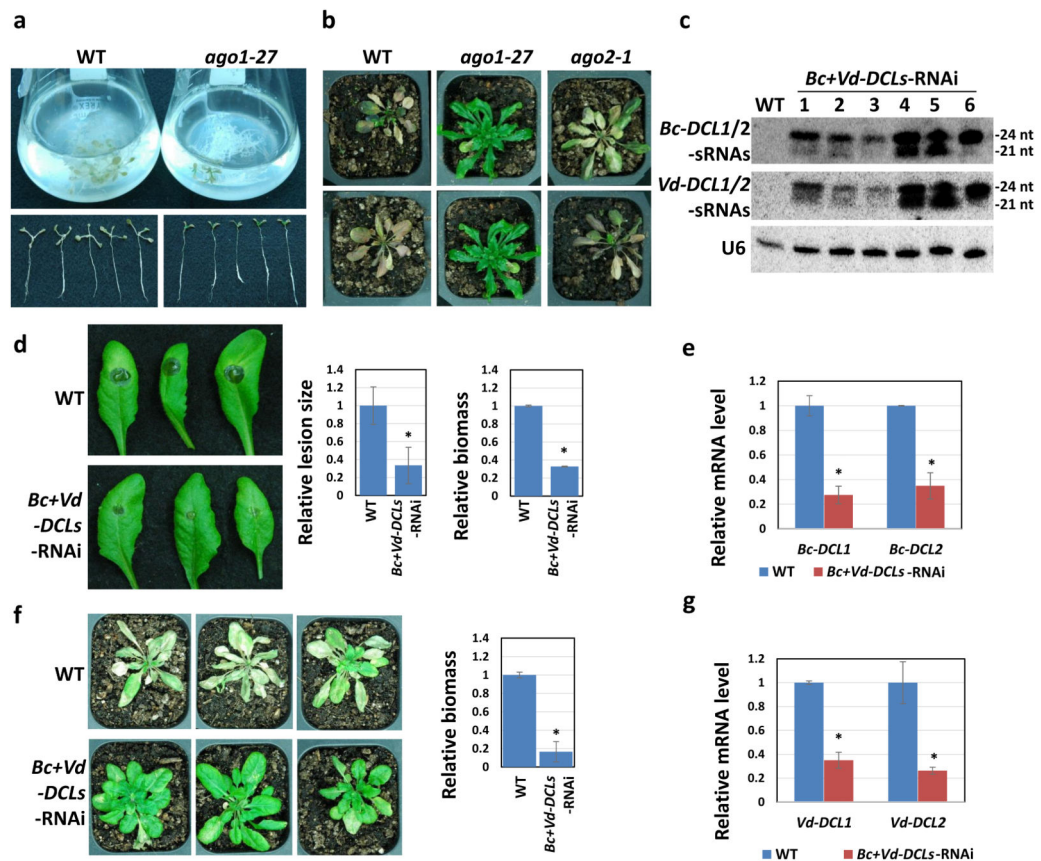
Figure 6. *Arabidopsis* **plants expressing hairpin RNAs that simultaneously target *DCL* genes of *B. cinerea* and *V. dahilae* show enhanced disease resistance to both pathogens**

(a) The *Arabidopsis ago1-27* mutant was more resistant to *V. dahilae* compared with WT plants in root culture conditions. (b) *Arabidopsis ago1-27*, but not *ago2-1* mutant, was less susceptible to *V. dahilae* when compared with 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 SD of 10 leaves. *B. cinerea* biomass was measured 3 dpi by quantitative PCR, and error bars indicate the SD of three technical replicates. (e) Quantitative RT-PCR showed that *Bc-DCL1* and *Bc-DCL2* were silenced in *B. cinerea*-infected *Arabidopsis Bc+Vd-DCLs*-RNAi plants compared with WT plants. (f) *Arabidopsis Bc+Vd-DCLs*-RNAi plants were less susceptible to *V. dahilae* compared to WT plants. Relative biomass of *V. dahilae* was measured at 3 weeks post inoculation, and error bars indicate the SD of three technical replicates. (g) The expression level of *Vd-DCL1* and *Vd-DCL2* were suppressed in *V. dahilae*-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).