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**Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways**

Arne Weiberg,1,2,3 Ming Wang,1,2,3 Feng-Mao Lin,1 Hongwei Zhao,1,2,3 † Zhizhong Zhang,1,2,3,5 Isgouhi Kaloshian,2,3,6 Hsien-Da Huang,1,7 Hailing Jin1,2,3,†

Botrytis cinerea, the causative agent of gray mold disease, is an aggressive fungal pathogen that infects more than 200 plant species. Here, we show that some *B. cinerea* small RNAs (Bc-sRNAs) can silence *Arabidopsis* and tomato genes involved in immunity. These Bc-sRNAs hijack the host RNA interference (RNAi) machinery by binding to *Arabidopsis Argonaute 1* (AGO1) and selectively silencing host immunity genes. The *Arabidopsis agao1* mutant exhibits reduced susceptibility to *B. cinerea*, and the *B. cinerea* dci1 dc12 double mutant that can no longer produce these Bc-sRNAs displays reduced pathogenicity on *Arabidopsis* and tomato. Thus, this fungal pathogen transfers "virulent" sRNA effectors into host plant cells to suppress host immunity and achieve infection, which demonstrates a naturally occurring cross-kingdom RNAi as an advanced virulence mechanism.

Botrytis cinerea is a fungal pathogen that infects almost all vegetable and fruit crops and annually causes $10 \times 10^9$ to $100 \times 10^9$ dollars in losses worldwide. With its broad host range, *B. cinerea* is a useful model for studying the pathogenicity of aggressive fungal pathogens. Many pathogens of plants and animals deliver effectors into host cells to suppress host immunity (1–4). All the pathogen effectors studied so far are proteins. We found that small RNA (sRNA) molecules derived from *B. cinerea* can act as effectors to suppress host immunity.

sRNAs induce gene silencing by binding to Argonaute (AGO) proteins and directing the RNA-induced silencing complex (RISC) to genes with complementary sequences. sRNAs from both plant and animal hosts have been recognized as regulators in host-microbial interaction (5–8). Although sRNAs are also present in various fungi and oomycetes, including many pathogens (9–14), it has not been clear whether they regulate host-pathogen interaction.

To explore the role of *B. cinerea* sRNAs in pathogenicity, we profiled sRNA libraries prepared from *B. cinerea* (strain B50.10)–infected *Arabidopsis thaliana* Col-0 leaves collected at 0, 24, 48, and 72 hours after inoculation and from *B. cinerea*–infected *Solanum lycopersicum* (tomato) leaves and fruits at 0, 24, and 72 hours after inoculation. sRNA libraries prepared from *B. cinerea* mycelia, conidiospores, and total biomass after 10 days of culture were used as controls. By using 100 normalized reads per million *B. cinerea* sRNA reads as a cutoff, we identified a total of 832 sRNAs that were present in both *B. cinerea*–infected *Arabidopsis* and *S. lycopersicum* libraries and had more reads in these libraries than in the cultured *B. cinerea* libraries, with sequences exactly matching the *B. cinerea* B05.10 genome (15) but not *Arabidopsis* or *S. lycopersicum* genomes or cdNA (tables S1 to S3). The closest sequence matches in *Arabidopsis* or *S. lycopersicum* contained a minimum of two mismatches. Among them, 27 had predicted microRNA (miRNA)–like precursor structures. A similar number of miRNA-like sRNAs were found in *Sclerotinia sclerotiorum* (9). We found that 73 Bc-sRNAs could target host genes in both *Arabidopsis* and *S. lycopersicum* under stringent target prediction criteria (tables S3). Among them, 52 were derived from six retrotransposon long terminal repeats (LTR) loci in the *B. cinerea* genome, 13 were from intergenic regions of 10 loci, and eight were mapped to five protein-coding genes.

Some of the predicted plant targets, such as mitogen-activated protein kinases (MAPKs), are likely to function in plant immunity. To test whether Bc-sRNAs could indeed suppress host genes during infection, three Bc-sRNAs (Bc-siR3.1, Bc-siR3.2, and Bc-siR5) were selected for further characterization (table S2). These Bc-sRNAs were among the most abundant sRNAs that were 21 nucleotides (nt) in length and had potential targets likely to be involved in plant immunity in both *Arabidopsis* and *S. lycopersicum*. These sRNAs were also enriched after infection (Fig. 1, A and B; fig. S1; and table S2) and were the major sRNA products from their encoding loci, LTR retrotransposons (fig. S1). Bc-siR3.1 and Bc-siR3.2 were derived from the same locus with a 4-nt shift in sequence.

To determine whether Bc-sRNAs could trigger silencing of host genes, we examined the transcript levels of the predicted target genes after *B. cinerea* infection. The following *Arabidopsis* genes were targeted in the coding regions and were suppressed after *B. cinerea* infection: mitogen activated protein kinase 2 (MPK2) and MPK1, which are targeted...
by Bc-siR3.2; an oxidative stress-related gene, peroxidase (PRXII), which is targeted by Bc-siR3.1; and cell wall-associated kinase (WAK), which is targeted by Bc-siR5 (Fig. 1C). In contrast, the plant defense marker genes PDF1.2 and BIK1 (16), which do not contain the Bc-sRNA target sites, were highly induced upon B. cinerea infection (Fig. 1C). We conclude that suppression of some but not all genes is a result of sequence-specific sRNA interaction and not due to cell death within infected lesions. Bc-siR3.2, which silences Arabidopsis MPK1 and MPK2, was enriched also in S. lycopersicum leaves upon B. cinerea infection (Fig. 1B) and was predicted to target another member of the MAPK signaling cascade in S. lycopersicum, MAPK4K4 (table S2). Expression of MAPKKK4 was indeed suppressed upon B. cinerea infection (Fig. 1D).

To confirm that the suppression of the targets was indeed triggered by Bc-sRNAs, we performed coexpression assays in Nicotiana benthamiana. Expression of hemagglutinin (HA)-epitope tagged MPK2, MPK1, and WAK was reduced when they were coexpressed with the corresponding Bc-sRNAs but not when coexpressed with Arabidopsis miR395, which shared no sequence similarity (Fig. 1E). The silencing was abolished, however, when the target genes carried a synonymously mutated version of the relevant Bc-sRNA target sites (Fig. 1E and fig. S2A). We also observed suppression of yellow fluorescent protein (YFP)-tagged target MPK2 by B. cinerea infection at 24 hours after inoculation (Fig. 1F and fig. S2B); when the Bc-siR3.2 target site of MPK2 was mutated, infection by B. cinerea failed to suppress its expression (Fig. 1F and fig. S2B). Thus, Bc-siR3.2 delivered from B. cinerea is sufficient for inducing silencing of wild-type MPK2 but cannot silence target site-mutated MPK2. Similarly, of the YFP-sensors with wild-type or mutated Bc-siR3.2 target sites (fig. S2C), only the wild-type sensor was suppressed after B. cinerea infection (Fig. 1G).

To test the effect of Bc-sRNAs on host plant immunity, we generated transgenic Arabidopsis plants that ectopically expressed Bc-siR3.1, Bc-siR3.2, or Bc-siR5 using a plant artificial miRNA vector (Fig. 2A) (17). These Bc-sRNA expression (Bc-sRNAox) lines showed normal morphology and development without pathogen challenge when compared with the wild-type plants, and expression of the target genes was suppressed (Fig. 2B). With pathogen challenge, all of the Bc-sRNAox lines displayed enhanced susceptibility to B. cinerea (Fig. 2, C and E). The Arabidopsis targets of Bc-siR3.2, MPK1 and MPK2, are homologs that share 87% amino acid identity. These genes are functionally redundant and are coactivated in response to various stress factors (18). The mpk1 mpk2 double mutant exhibited enhanced susceptibility to B. cinerea (Fig. 2, D and E). A transferred-DNA knockout mutant of the Bc-siR5 target WAK (SALK_089827) (fig. S3A) also displayed enhanced susceptibility to B. cinerea (Fig. 2, D and E).

**Fig. 1.** Bc-sRNAs silence host target genes in both Arabidopsis and S. lycopersicum during B. cinerea infection. (A) Bc-siR3.1, Bc-siR3.2, and Bc-siR5 were expressed during infection of Arabidopsis as detected at 18, 24, 48, and 72 hours after inoculation and (B) S. lycopersicum leaves at 18, 24, 32, 48 hours after inoculation by means of reverse transcription polymerase chain reaction (RT-PCR). Actin genes of B. cinerea, Arabidopsis, and S. lycopersicum were used as internal controls. Similar results were obtained from three biological replicates. (C) The Arabidopsis targets of Bc-sRNAs were suppressed after B. cinerea infection. PDF1.2, BIK1, and β-tubulin were used as controls. (D) The S. lycopersicum target gene MAPKKK4 was suppressed upon B. cinerea infection. Expression [(C) and (D)] was measured by means of quantitative RT-PCR by using actin as an internal control. Error bars indicate SD of three technical replicates. (E) Coexpression of Bc-siR3.2 or Bc-siR5 with their host targets (HA-tagged) in N. benthamiana revealed target silencing by means of Western blot analysis. Coexpression of AtmiR395 or target site–mutated versions of target genes was used as controls. (F) Expression of YFP-MPK2 or its synonymously mutated version (YFP-MPK2-m) after infection of B. cinerea was observed with confocal microscopy. Coexpression of the YFP-MPK2 and Bc-siR3.2 was used as a control. (G) Expression of the YFP sensors carrying a Bc-siR3.2 target site of MPK2 or a Bc-siR3.2 target site-m was analyzed after infection of B. cinerea. Samples were examined at 24 hours after inoculation. (Top) YFP. (Bottom) YFP/bright field overlay. Scale bars [(F) and (G)], 37.5 μm. Error bars indicate SD of 20 images [(F) and (G)]. The asterisk indicates significant difference (two-tail t-test; P < 0.01). Similar results were obtained in three biological replicates in (E) to (G).
with this, Bc-sRNAox lines as well as mpk1 mpk2 and wak showed lower induction of the defense marker gene BIK1 (fig. S3B). These results suggest that the MPK1, MPK2, and WAK genes, all of which are targeted by Bc-sRNAs, participate in the plant’s immune response to B. cinerea. To determine whether MAPKKK4 is involved in S. lycopersicum defense response against B. cinerea, we applied the virus-induced gene silencing (VIGS) approach to knock down MAPKKK4 in S. lycopersicum using tobacco rattles virus (TRV) (fig. S4A) (19). VIGS of TRV-MAPKKK4 caused a dwarf phenotype (fig. S4B).

The MAPKKK4-silenced plants showed enhanced disease susceptibility in response to B. cinerea and contained >15 times more fungal biomass than that of the control plants (Fig. 2F). We conclude that Bc-sRNAs silence plant genes to suppress host immunity during early infection.

These fungal sRNAs hijack the plant’s own gene silencing mechanism. Sixty-three of the 73 Bc-sRNAs that had predicted Arabidopsis and S. lycopersicum targets were 20 to 22 nt in length with a 5′ terminal U (table S3). This sRNA structure is favored for binding to AGO1 in Arabidopsis (20, 21). In order to determine whether Bc-sRNAs act through Arabidopsis AGO1, we immunoprecipitated AGO1 from B. cinerea–infected Arabidopsis collected at 24, 32, and 48 hours after inoculation and analyzed the AGO1-associated sRNAs. Bc-siR3.1, Bc-siR3.2, and Bc-siR5 were clearly detected in the AGO1-associated fraction pulled down from the infected plant samples but hardly in the control (Fig. 3A) or in the AGO2- and AGO4-associated sRNA fractions (fig. S5). The sRNAs that had no predicted plant targets or had predicted targets that were not downregulated by B. cinerea infection were not found in the AGO1-associated fractions (fig. S6).

Fig. 2. Bc-sRNAs trigger silencing of host targets that are involved in host immunity. (A) Expression of Bc-siR3.1, Bc-siR3.2, or Bc-siR5 in transgenic Arabidopsis ectopically expressing Bc-sRNAs under the Cauliflower Mosaic Virus promoter 35S (Bc-sRNAox) was examined by means of Northern blot analysis. Highly expressed lines were selected for the following experiments. (B) Bc-sRNAox lines showed constitutive silencing of respective Bc-sRNA target genes measured with quantitative RT-PCR. Two independent lines for each Bc-sRNA were examined. Similar results were observed in two generations of the selected transgenic lines. (C) Bc-sRNAox plants exhibited enhanced disease susceptibility to B. cinerea as compared with wild type. (D) Loss-of-function mutants of Bc-siR3.2 and Bc-siR5 targets mpk1 mpk2 and wak displayed enhanced disease susceptibility. In all pathogen assays [(C) and (D)], lesion sizes were measured at 96 hours after inoculation. Error bars indicate the SD of 20 leaves. (E) Biomass of B. cinerea was measured with quantitative PCR at 96 hours after inoculation. Error bars indicate SD of three technical replicates. For (C), (D), and (E), similar results were obtained from three biological repeats. (F) VIGS of MAPKKK4 exhibited enhanced disease susceptibility to B. cinerea in S. lycopersicum (examined at 72 hours after inoculation) as compared with control plants (TRV-RB). RB is a late-blight resistance gene that is not present in tomato. We chose to use a TRV vector with a fragment from a foreign gene as a control to eliminate the potential side effect of viral disease symptoms caused by TRV empty vector. Spray inoculation was used because silencing sectors are not uniform within the VIGS plants. Three sets of experiments with each of 6 to 10 plants for each construct were performed, and similar results were obtained. The asterisk indicates significant difference (two-tail t-test, P < 0.01) in (C) to (F).
If AGO1 plays an essential role in Bc-sRNA-mediated host gene silencing, we would expect to see reduced disease susceptibility in the ago1 mutant because the Bc-sRNAs could no longer suppress host immunity genes. For plants carrying the ago1-27 mutant allele and were inoculated with B. cinerea, the disease level was significantly less than on the wild type (Fig. 3B and fig. S7A). Consistent with this, BIK1 induction was increased compared with that of the wild-type (fig. S7B). Furthermore, the expression of Bc-siR3 target MPK2 and MPK1, Bc-siR3.1 target PRXII, and Bc-siR5 target WAK in ago1-27 was not suppressed compared with those in wild-type infected plants after B. cinerea infection (Fig. 3C). The expression of Bc-siR3.1, Bc-siR3.2, and Bc-siR5 could not be detected in the ago1-27 mutant (Fig. 3A), indicating that they were DCL-dependent, whereas two other Bc-sRNAs, Bc-miR2 and Bc-siR1498, could still be detected in dcl1 dcl2 double mutant (fig. S9D). Fungi have diverse sRNA biogenesis pathways, and not all sRNAs are DCL-dependent (12). The dcl1 dcl2 double mutant caused significantly smaller lesions than those of the wild type or dcl1 and dcl2 single mutants on both Arabidopsis and S. lycopersicum leaves (Fig. 4, B and C), in consistence with the significantly reduced fungal biomass at 72 hours after inoculation in Arabidopsis and 48 hours after inoculation in S. lycopersicum (fig. S10), which indicates that the virulence of the dcl1 dcl2 mutant was greatly reduced. These results further support the conclusion that Bc-sRNAs—particularly Bc-siR3.1, Bc-siR3.2, and Bc-siR5, which depend on B. cinerea DCL function—contribute to the pathogenicity of B. cinerea. Mutation of dcl1 or dcl2 in B. cinerea caused delayed growth and sporulation (fig. S9C) but had no effect on pathogenicity (Fig. 4, B and C). Furthermore, expression of the YFP sensor carrying the Bc-siR3.2 target site in N. benthamiana was silenced when infected much as that in B. cinerea–infected leaves at 48 hours after inoculation (48 hpi) were used to rule out any binding between AGO1 and Bc-sRNAs during the experimental procedures. Similar results were obtained from at least three biological repeats. (B) Arabidopsis ago1-27 exhibited reduced disease susceptibility to B. cinerea as compared with the wild type. Lesion size of at least 20 leaves and fungal biomass were measured at 96 hpi after inoculation. (C) Silencing of MPK2, MPK1, PRXII, and WAK during B. cinerea infection was abolished in ago1-27. (D) Arabidopsis dcl1-7 exhibited enhanced disease susceptibility to B. cinerea as compared with the wild type. Similar results were obtained from three biological repeats ([B] to [D]). The asterisk indicates significant difference (two-tail t-test, P < 0.01) in (B) and (D).
with wild-type *B. cinerea*. The suppression was abolished when inoculated with the *dcl1 dcl2* strain (Fig. 4D), indicating that the *dcl1 dcl2* double mutant was unable to generate Bc-siR3.1, Bc-siR3.2, and Bc-siR5 as revealed with RT-PCR. *B. cinerea dcl1 dcl2* double mutant, but not *dcl1* or *dcl2* single mutants, produced much weaker disease symptoms than did the wild type in (B) *Arabidopsis* and (C) *S. lycopersicum*, as demonstrated by the lesion size measured of 20 leaves at 96 and 48 hours after inoculation, respectively. Similar results were obtained from three biological repeats. (D) Expression of the sensor YFP-Bc-siR3.2 target site was silenced by wild-type *B. cinerea* upon infection, but not by the *dcl1 dcl2* mutant at 24 hours after inoculation. Scale bar, 75 µm. Error bars indicate SD of 20 images. Experiments were repeated two times with similar results. 

Animal and plant pathogens have evolved virulence or effector proteins to counteract host immune responses. Various protein effectors have been predicted or discovered in fungal or oomycete pathogens from whole-genome sequencing and secretome analysis (2, 3), although delivery mechanisms are still under active investigation (23–27). Here, we show that sRNAs as well can act as effectors through a mechanism that silences host genes in order to debilitate plant immunity and achieve infection. The sRNAs from *B. cinerea* hijack the plant RNAi machinery by binding to AGO proteins, which in turn direct host gene silencing. Another fungal plant pathogen, *Verticillium dahliae*, also depends on AGO1 function for its pathogenicity (28). The implications of these findings may extend beyond plant gray mold disease caused by *B. cinerea* and suggest an extra mechanism underlying pathogenesis promoted by sophisticated pathogens with the capability to generate and deliver small regulatory RNAs into hosts to suppress host immunity.

### References and Notes

Crystal Structure of Na\(^{+}\), K\(^{+}\)-ATPase in the Na\(^{+}\)-Bound State

Maria Nyblom,1,2,‡‡ Hanne Poulsen,1,2,3‡‡ Pontus Gourdon,1,2,3§ Linda Reinhard,1,2‡§ Magnus Andersson,4 Erik Lindahl,5,6 Natalya Fedosova,1,4 Poul Nissen1,2,3‡

The Na\(^{+}\), K\(^{+}\)—adenosine triphosphatase (ATPase) maintains the electrochemical gradients of Na\(^{+}\) and K\(^{+}\) across the plasma membrane—a prerequisite for electrical excitability and secondary transport. Hitherto, structural information has been limited to K\(^{+}\)-bound or ouabain-blocked forms. We present the crystal structure of a Na\(^{+}\)-bound Na\(^{+}\), K\(^{+}\)-ATPase as determined at 4.3 Å resolution. Compared with the K\(^{+}\)-bound form, large conformational changes are observed in the α subunit whereas the β and γ subunit structures are maintained. The locations of the three Na\(^{+}\) sites are indicated with the recent X-ray structure of the recently published structure of αII, further supported by electrophysiological studies on leak currents. Extracellular release of the third Na\(^{+}\) from αII through the open gate of the ATPase is suggested at sites I and II of the α subunit.

The Na\(^{+}\), K\(^{+}\)—adenosine triphosphatase (ATPase) is typically a ternary complex of a catalytic α subunit associated with two smaller subunits, β and γ (Fig. 1A). Different isoforms combine to form kinetically distinct complexes in different cells and tissues (1). During the ATP-driven transport cycle, three cytoplasmic Na\(^{+}\) are exported in exchange for two extracellular Na\(^{+}\) and K\(^{+}\) across the plasma membrane (Fig. 1B), where E1 and E2 denote high affinity for Na\(^{+}\) and high-affinity Mg\(^{2+}\) (E2P) states, respectively. The third Na\(^{+}\) site has remained unsettled.

The ability of the E1-AlF\(_4\)-ADP complex to occlude three Na\(^{+}\) under crystallization-like conditions was confirmed by time-course measurements of 22Na\(^{+}\) deocclusion at 0°C (Fig. 1F). The monoexponential fit resulted in the maximal number of 2.5 nmol of Na\(^{+}\) per nmol of ADP binding sites (i.e., 83% occupancy) at 1 mM Na\(^{+}\) and the deocclusion rate constant of 0.02 s\(^{-1}\). Assuming a Hill coefficient of 3 for the cooperative Na\(^{+}\) binding, the ion concentration required for the half-maximal saturation of the sites (K\(_{0.5}\) for Na\(^{+}\)) was calculated to be 0.58 mM, consistent with previous findings (13). Thus, Na\(^{+}\) concentration under crystallization conditions (>80 mM) was more than two orders of magnitude higher than K\(_{0.5}\), enough to saturate all three sites.

The α subunit represents a Na\(^{+}\)—bound form of the transmembrane (TM) domain, with the cytoplasmic A, P, and N domains arranged for phosphorylation (Fig. 1, C and D) as observed for sarcoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA1a) in the equivalent, Ca\(^{2+}\)-occluded state (Fig. S4). The ability of the E1-AlF\(_4\)-ADP complex to occlude three Na\(^{+}\) under crystallization-like conditions was confirmed by time-course measurements of 22Na\(^{+}\) deocclusion at 0°C (Fig. 1F). The monoexponential fit resulted in the maximal number of 2.5 nmol of Na\(^{+}\) per nmol of ADP binding sites (i.e., 83% occupancy) at 1 mM Na\(^{+}\) and the deocclusion rate constant of 0.02 s\(^{-1}\). Assuming a Hill coefficient of 3 for the cooperative Na\(^{+}\) binding, the ion concentration required for the half-maximal saturation of the sites (K\(_{0.5}\) for Na\(^{+}\)) was calculated to be 0.58 mM, consistent with previous findings (13). Thus, Na\(^{+}\) concentration under crystallization conditions (>80 mM) was more than two orders of magnitude higher than K\(_{0.5}\), enough to saturate all three sites.

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