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

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*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* ago1 mutant exhibits reduced susceptibility to *B. cinerea*, and the *B. cinerea del1 del2* 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.

*B. cinerea* is a fungal pathogen that infects almost all vegetable and fruit crops and annually causes $10 billion to $100 billion 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 of host-pathogen 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 B05.10-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* sequences 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, peroxiredoxin (PRXIII), 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, MAPKKK4 (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). 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 results indicate that these Bc-sRNAs play a positive role in B. cinerea pathogenicity.

Enhanced disease susceptibility of the Bc-sRNAox lines suggests that the target genes of these Bc-sRNAs are likely to be involved in host immunity against B. cinerea. Plants with mutated target genes showed normal morphology and development without pathogen challenge. 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). Consistent
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 rat-tail 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 sRNA fractions (fig. S5). The sRNAs that had no predicted plant targets or had predicted targets that were not down-regulated 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 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 these Bc-sRNAs could no longer suppress host immunity genes. For plants carrying the ago1-27 mutant allele (22) 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, BIKI induction was increased compared with that of the wild-type (fig. S7B). Furthermore, the expression of Bc-siR3.2 targets MPK2 and MPK1, Bc-siR3.1 target PRXIIIF, 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). On the contrary, *Arabidopsis* miRNA biogenesis mutant dicer-like (dcl) 1-7 that shows similar morphological defects to ago1-27 exhibited an enhanced disease level to *B. cinerea* (Fig. 3D). These results suggest that the increased resistance phenotype we observed in ago1-27 is not caused by any reduced vigour or pleiotropic phenotype but was due to the function of the Bc-sRNAs, and that *Arabidopsis* DCL1 is not required for the function of Bc-sRNAs. Thus, Bc-sRNAs evidently hijacked host RNAi machinery by loading into AGO1; the complex in turn suppressed host immunity genes.

To delete the siR3 and siR5 loci from the *B. cinerea* genome by homologous recombination would be an ideal way to confirm their function; however, it is not feasible because siR3 is from a LTR with three copies and siR5 is from a LTR with 13 copies. To better understand the function and biogenesis of the Bc-sRNAs, we chose to knock out the *B. cinerea* DCL1 genes, which encode the core sRNA processing enzymes. *B. cinerea* strain B05.10 possesses two Dicer-like genes (*Bc-DCL1* and *Bc-DCL2*) (fig. S8). We generated *dcl1* and *dcl2* single and *dcl1 dcl2* double knockout mutant strains through homologous recombination (fig. S9, A and B). We found that *dcl1* and *dcl2* single mutants showed reduced growth and delayed sporulation (fig. S9C). The *dcl1 dcl2* double mutant displayed a more obvious phenotype than that of each of the single mutants, suggesting partial functional redundancy between DCL1 and DCL2 in *B. cinerea*. Bc-siR3.1, Bc-siR3.2, and Bc-siR5 could not be detected in the *dcl1 dcl2* double mutant (Fig. 4A), indicating that they were DCL-dependent, whereas two other Bc-sRNAs, Bc-miR12 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 consistency 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, PRXIIIF, 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-siR3.2 to suppress the target. We also confirmed the inability of dcl1 dcl2 to suppress Bc-siR3.1 and Bc-siR3.2 target genes MPK2, MPK1, and PRXIIF in Arabidopsis and MAPKKK4 in S. lycopersicum. Similar results were seen in two biological repeats. Infection of Arabidopsis Bc-siR3.1ox and Bc-siR3.2ox lines was more susceptible to B. cinerea dcl1 dcl2 strain than was Col-0 wild type. Enhanced disease phenotype of dcl1 dcl2 infection was also observed on TRV-MAPKKK4–silenced S. lycopersicum plants. Experiments in (F) and (G) were repeated three times with similar results. B. cinerea biomass was quantified at 96 hours after inoculation. The asterisk [in (B), (C), (D), (F), and (G)] indicates significant difference (two-tail t-test; P < 0.01).

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 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
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 transport. Hitherto, structural information has been limited to K+-bound or ouabain-blocked forms.

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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 unique site III at the recently suggested IIb, as further supported by electrophysiological studies on leak currents. Extracellular release of the third Na+ from IIb through IIa, followed by exchange of Na+ for K+ at sites I and II, is suggested.

The Na+, K+-adenosine triphosphatase (ATPase) is typically a tetrameric complex of a large 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 (I). During the ATP-driven transport cycle, three cytoplasmic Na+ are exported in exchange for two extracellular Na+ and K+ across the plasma membrane—primarily at the Na+/K+ pump, ATP-driven Na+/K+-dependent transport, and Na+/Ca2+ exchange. In addition, Na+−K+-adenosine triphosphatase (Na+, K+-ATPase) is involved in the maintenance of extracellular Na+ concentrations and intracellular K+ concentrations, as well as in the regulation of cellular pH and the generation of a transmembrane electrical potential.

In the Na+, K+−adenosine triphosphatase (ATPase), three Na+ binding sites (i.e., 83% occupancy) at 1 mM22Na+ are occupied, with a Hill coefficient of 3 for the cooperative binding of Na+. The Hill coefficient of 3 for the cooperative binding of Na+ is consistent with previous findings (13). Thus, Na+ concentration under crystallographic conditions (>80 mM) was more than two orders of magnitude higher than the Kd,5 enough to saturate all three sites.

The α subunit represents a Na+-saturating 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 sarco(endo)plasmic reticulum Ca2+ ATPase 1a (SERCA1a) in the equivalent, Ca2+-occluded state (Fig. 5B) (14–16). Thus, compared with K+-bound forms, the α subunit is characterized by a different organization of the TM helices and a compact configuration of the cytoplasmic domains activating the phosphorylation site (Fig. 1, C and D, and fig. S5). Relative to the P domain, the A domain has undergone a rigid-body rota-

We present here the crystal structure determined at 4.3 Å of the Na+, K+-ATPase in the [Na3]E1P-ADP state (pig renal α1βγ enzyme) as mimicked by an ADP-AIF4− complex (materials and methods and table S1) for which Na+ saturation was further stabilized by the presence of oligomycin. The structure was determined from an unbiased electron density map derived by single isomorphous replacement with anomalous scattering (SIRAS), using hexatantalum dodecabromide (Ta6Br12) derivatized crystals, followed by density modification procedures (Fig. 1C and fig. S2). Model building using sharpened maps and restrained refinement produced a final model with Rwork and Rfree of 26.1 and 28.6%, respectively. The structure represents two nearly identical complexes in the asymmetric unit (chains A-B-G and C-D-E) and displays bilayer features in the electron density (figs. S2 to S4).

The ability of the E1-AIF4−-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 mM22Na+ 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 (K0.5 for Na+) was calculated to be 0.58 mM, consistent with previous findings (13). Thus, Na+ concentration under crystallographic conditions (>80 mM) was more than two orders of magnitude higher than the K0.5, enough to saturate all three sites.

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Crystal Structure of Na+, K+-ATPase in the Na+-Bound State

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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 unique site III at the recently suggested IIb, as further supported by electrophysiological studies on leak currents. Extracellular release of the third Na+ from IIb through IIa, followed by exchange of Na+ for K+ at sites I and II, is suggested.