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The Role of Fungal Pathogen Small RNAs in Host-Microbe Interactions

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by

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ABSTRACT OF THE DISSERTATION

The Role of Fungal Pathogen Small RNAs in Host-Microbe Interactions

by

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Botrytis cinerea is a necrotrophic fungal pathogen that causes gray mold disease on a broad range of plant species. Many pathogens secrete protein effectors into host cells to evade the host immune system; however, my dissertation project shows that *B. cinerea* small RNAs (Bc-sRNAs) act as a novel type of pathogen effector to silence host defense genes.

Small RNAs (sRNAs) are short non-coding RNAs that normally associate with Argonaute (AGO) protein and suppress the genes with complementary sequences. The role of host sRNAs in plant-pathogens interactions has been well characterized, and recent studies also revealed the function of pathogen sRNAs in infection processes. In the first chapter of this thesis, I will review the current progress of the role of both host sRNAs and microbial pathogen sRNAs during host-pathogen interactions.

Bc-sRNA effectors are induced during plant infection and trigger silencing of host plant genes. We identified and confirmed three Bc-sRNAs (Bc-siR3.1, Bc-siR3.2 and Bc-siR5) that can translocate into host cells and hijack host RNAi machinery to silence host

immunity related target genes. The *B. cinerea dcl1 dcl2* double mutant has lost Bc-siR3.1, Bc-siR3.2, and Bc-siR5, which significantly compromised its virulence. The second chapter will present these findings.

Chapter 3 will cover the identification and characterization of a new Bc-sRNA effector, Bc-siR37, which has multiple predicted target genes in both Arabidopsis and tomato (*Solanum lycopersicum*), and most are putatively related to plant defense. We further characterize three of these candidate targets, *At-WRKY7*, *At-PMR6*, and *At-FEI2*, and confirm that they are negatively correlated with Bc-siR37 and positively regulate plant immunity against *B. cinerea*.

Comparative analysis of Bc-sRNAs transcriptome in wild-type *B. cinerea* and the *dcl1 dcl2* double mutant indicates that most retrotransposon region-derived Bc-sRNAs are DCL-dependent, and most predicted Bc-sRNA effectors are generated from retrotransposon regions. The compromised virulence of the *B. cinerea dcl1 dcl2* double mutant is probably due to failure to produce many Bc-sRNA effectors. Finally, we successfully use host-induced gene silencing (HIGS) of *B. cinerea DCL1* and *DCL2* to enhance plant resistant against gray mold disease. The final chapter will focus on these results.

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Chapter 1

The role of small RNAs in host plants and microbial pathogens interactions

Abstract

Small RNAs (sRNAs) are 20–30 nucleotide (nt) non-coding RNAs that are normally processed by type III endoribonuclease Dicer or Dicer-like (DCL) proteins. After production, sRNAs associate with Argonaute (AGO) protein and form a RNA-induced Silencing Complex (RISC). sRNAs can guide the RISC to its targets by sequence complementarity, and the RISCs can then silence target genes through mRNA cleavage or translation inhibition (post-transcriptional) or chromatin modification or DNA methylation (transcriptional). The function of host sRNAs in host-pathogen interactions has been well investigated, and recent studies also indicated the role of pathogen sRNAs in this processes. This chapter will summarize the role of both host sRNAs and microbial pathogen sRNAs during host-pathogen interactions.

Introduction

Over recent decades, profound findings in plant pathology research have made tremendous contributions to our understanding of how pathogens are able to colonize the biological niche of a living plant. Genetic approaches have determined pathogenicity or virulence factors, and the exploration of these factors has broadened our understanding of host–pathogen interactions. A group of virulence genes that code for secreted proteins are

called effectors, and have received much attention, because effectors interfere with and manipulate host defense pathways for infection[1-4].

To counter against pathogen effectors, host plants evolved resistance (R) gene-encoding proteins to interact directly or indirectly with pathogen effectors, which mount a strong immune reaction, a process called effector-triggered immunity (ETI). An evolutionary arms race occurs between hosts and pathogens, which drive the pathogens to reinvent their effector molecules to undermine host plant immunity, and drives the hosts to update their molecular immune fence line to recognize effectors and to defeat pathogens by intensifying its immune response [5-8].

RNA interference (RNAi) or gene silencing is a mechanism in which small RNAs (sRNAs) guide the transcriptional and posttranscriptional silencing of gene expression. It is an ancient and conserved mechanism present in almost all eukaryotic life forms, including plants, animals, fungi and oomycetes [9,10]. sRNAs are classified into three major groups, microRNAs (miRNAs), small interference RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). piRNAs exclusively exist in animals, while both miRNAs and siRNAs widely exist in almost all eukaryotes. miRNAs are generated from the *MIR* gene encoded primary miRNAs (pri-miRNAs), which form the stem-loop hairpin structures; whereas, siRNAs are processed from long double strand RNAs (dsRNAs). Typically, the pri-miRNAs and the long dsRNAs are mostly digested by Dicer or Dicer-like proteins (DCLs) into mature miRNAs and siRNAs, respectively [11]. The mature sRNAs are loaded into Argonaute (AGO) proteins, and form the RNA-induced silencing complex (RISC) [12]. The RISC silences genes with complementary sequences to sRNAs [13-15]. RNAi and

sRNAs are important players in defence against viruses and other invading DNA elements, such as transposable elements (TE) and transgenes [16,17]. Moreover, sRNAs also play an important role in the regulation of the expression of endogenous genes. Gene silencing occurs in diverse cellular processes, including plant defense pathways against various pathogen attacks [18-20].

The regulatory role of plant endogenous sRNAs in plant innate immunity has been studied intensively, which include anti-virus, anti-bacteria, anti-oomycete and anti-fungi processes [17,19,21,22]. The development and improvement of next generation deep sequencing techniques tremendously help the discovery of plant immunity related sRNAs. Recent evidence has also demonstrated the important roles of pathogen-derived sRNAs in host–microbe interactions, and these pathogen sRNAs were named sRNA effectors [23,24]. Similar to protein effectors, which usually are located near TEs, most of sRNA effectors are generated directly from TE. This feature facilitates the fast turnover of the effectors during host-pathogen co-evolution [19,23,25].

This chapter will discuss the roles of siRNAs and miRNAs from both host plants and microbial pathogens during their interactions, in particular addressing the roles of bacterial, oomycetes and fungal microbial pathogens. In addition, the cross-kingdom binary movement and function of sRNAs between plants and pathogens will also be summarized.

The function of host endogenous sRNAs in plant immunity against microbial pathogens (bacteria, oomycetes, and fungi).

In antibacterial defense responses

Arabidopsis miR393 is the first plant miRNA that was discovered to respond to biotic stress caused by infection with virulent bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 [21,22]. miR393 is triggered by a pathogen-associated molecular pattern (PAMP) flg22, a 22-amino acid peptide derived from the N terminus of bacterial flagellin, and it silences auxin receptors TIR1, AFB2, and AFB3 to suppress the auxin pathway. The inhibition of the auxin pathway enhances the plant defense response to *P. syringe*, indicating the positive role of miR393 in PAMP- triggered immunity (PTI) [22]. Interestingly, miR393*, the complementary strand of miR393, is induced by an avirulent bacterial strain *Pst* carrying the effector avrRpt2 and is loaded into AGO2 to target a golgi-localized SNARE protein MEMB12. The suppression of MEMB12 leads to increased exocytosis of the *Arabidopsis* antimicrobial pathogenesis-related protein PR1 [26]. In addition, *Arabidopsis* miR160, miR167, which target genes involved in the auxin pathway, are induced upon treatment with flg22 or infection with a *Pst* DC3000 strain that has a mutated type III secretion system (*hrcC*), an empty vector, or effector avrRpt2 [27,28]. flg22 treatment also causes the downregulation of AGO-associated *Arabidopsis* miR398b and miR773, overexpression of which decreases plant callose deposition during bacterial infection, indicating their negative role in plant immunity [28]. *Arabidopsis* miR400 is downregulated after infection by both non-pathogenic *Pst* DC3000 *hrcC* mutant and virulent strain *Pst* DC3000 [27,29] and regulates plant immunity against both bacterial

pathogen *Pst* DC3000 and fungal pathogen *B. cinerea* by cleaving two pentatricopeptide repeat encoding genes [30]. Similarly, *Arabidopsis* miR844 also acts as a negative regulator during plant defense to *Pst* DC3000 and *B. cinerea* by silencing a plant immune gene *CYTIDINEPHOSPHATE DIACYLGLYCEROL SYNTHASE 3* (*CDS3*) [31]. The infection of citrus by bacteria *Candidatus Liberibacter*, the causal agent of citrus Huanglongbing disease, induces citrus miRNA csi-miR399, which suppresses the ubiquitin-conjugating enzyme (*PHO2*)-encoding targets and regulates phosphorus homeostasis [32].

In addition, some plant miRNAs can guide the cleavage of multiple nucleotide-binding site leucine-rich repeat (NBS-LRR) immune receptors trigger secondary phased siRNAs (phasiRNAs) production. Most plant *R* genes encode NBS-LRR proteins. Therefore, miRNAs guided silencing of NBS-LRR type *R* genes during pathogen infection also contributes to plant immunity. The family of NBS-LRR encoding genes is subdivided into two subfamilies based on their distinct N terminal domain, which are toll and interleukin-1 receptor NBS-LRRs (TNLs) and coiled-coil NBS-LRRs (CNLs). When a plant is under microbial pathogens attack, suppression of these NBS-LRR type *R* genes by miRNAs is released, thus conferring strong plant defense responses against the pathogens. *Arabidopsis* miR472 was the first miRNA predicted to target a CNL gene and initiate the generation of phased siRNAs [33]. In the model legume species *Medicago truncatula*, miR1507, miR2109, and miR2118 are also predicted to target multiple *NBS-LRR* genes to initiate phasiRNAs accumulation [34]. The first experimentally characterized example of a plant miRNA regulating the expression of a *R* gene was conducted on tobacco plant

Nicotiana benthamiana. nta-miR6019 and nta-miR6020 negatively regulate the tobacco TNL gene *N,* which is an important resistance gene against tobacco mosaic virus (TMV). At the same time, the accumulation of 21-nt secondary siRNAs is triggered at the target site of *N*. The expression of nta-miR6019 and nta-miR6020 interferes with the tobacco *N* gene-mediated resistance to TMV [35]. In tomato (*Solanum lycopersicum*), miR482 also guides the cleavage of many CNL genes and initiates the production of 21-nt siRNAs. When tomato plants are infected by bacterial or viral pathogens, miR482-directed *R* gene silencing is suppressed, which allows the accumulation of R proteins to strengthen plant immune responses [36]. *Arabidopsis* miR472, which only has 2-nucleotide difference with tomato miR482, was further proved to regulate CNL-type *R* genes, including *RPS5* that recognizes the bacterial effector AvrPphB. Interestingly, *Arabidopsis* miR472 negatively regulates both PTI and ETI [37].

There are more examples of plant miRNA-directed *R* gene repression after challenging with microbial pathogens, especially the filamentous eukaryotic pathogens, and it will be discussed in next anti-fungi section. It is likely that such regulation is conserved among various plant species for disease resistance. They either directly or indirectly interact with pathogen effectors to turn on strong plant defense responses to limit the proliferation of pathogens. miRNA regulation is one of the indirect pathways to alter the expression of *R* genes and activate plant immunity. However, the question remains, what is the function of those phasiRNAs? In tomato, at least one of the phasiRNAs that target *R* gene loci was predicted to be targeted by a defense related gene [36]. Soybean pahsiRNAs in NBS-LRR loci are predicted to target additional NBS-LRR genes *in trans*

[38,39]. Moreover, the loss-of-function *Arabidopsis rdr6* mutant, an RNA-dependent RNA polymerase that is involved in secondary siRNA generation, enhanced plant antibacterial immunity at both PTI and ETI levels, indicating that *RDR6*-dependent phasiRNAs might participate in plant immunity [37]. However, more experiments are needed to confirm the role of the phasiRNAs in plant immunity.

Additionally, plant siRNAs also contribute to plant immunity against plant bacterial diseases. When *Arabidopsis* is infected by of avirulent *Pst* (*avrRpt2*), two native siRNAs, *Arabidopsis* nat-siRNAATGB2 and *Arabidopsis* lsiRNAs-1 (AtlsiRNA-1), are highly induced. Because they are formed from the overlapping regions of two genes, both of them aim to silence one of the overlapping genes. nat-siRNAATGB2, generated from the overlapping regions of the Rab2-like small GTP-binding protein gene (*ATGB2*) and pentatricopeptide repeats (*PPR*) protein-like gene (*PPRL*), is specifically induced by *Pst* (*avrRpt2*). It positively regulates plant immunity through silencing *PPRL*, a negative regulator of *RPS2*-mediated plant immunity [21]. Another example of plant anti-bacterial siRNA is AtlsiRNA-1, which is derived from the overlapping region of small RNA-generating receptor-like kinase (*SRRLK*) and a RNA-binding domain containing gene (*AtRAP*). AtlsiRNA-1 is about 40 nt in length, is also induced by *Pst* (*avrRpt2*), leading to the suppression of *AtRAP* by mRNA 5' decapping and induction of defense responses to both virulent *Pst* and avirulent *Pst* (*avrRpt2*) strains [40]. In addition to miR393*, three *Arabidopsis* sRNAs have been identified to regulate plant defense responses against *Pst* (*avrRpt2*) [41], indicating that multiple plant sRNAs work cooperatively to enhance plant immunity during pathogen infection.

In anti-oomycete pathogen responses.

Phytothphora sojae is a soil-borne plant pathogen that causes root rot on soybeans and is the second most destructive disease of soybean [42]. Originally, microarray data from *P. sojae*-infected soybean indicated the alteration of several soybean miRNAs, indicating that that soybean sRNAs play a role during plant defense responses to *P. sojae* [43,44]. Since then, Wong and coauthors performed global profiling of soybean plants with both resistant and sensitive cultivars (Williams with *Rps1-k*) challenged by *P. sojae.* Their findings indicated that miR166, miR393, miR1507, miR2109, and miR3522 were induced, while miR168, miR319, and miR482 were downregulated. MiR393 was further characterized to participate in plant immunity against *P. sojae* by positively regulating the soybean antimicrobial metabolites, e.g. the biosynthesis of isoflavonoid. miR1507 and miR2109 silenced NBS-LRR genes and triggered the generation of phasiRNAs, which were predicted to regulate more NBS-LRR genes [39]. However, it is still a mystery why *P. sojae* treatment cause suppression of NBS-LRR genes which supposed to be positive regulators of plant immunity. In fact, a recent research on global profiling of soybean sRNA showed that most of the soybean miRNA were down regulated yet most of their targets, the NBS-LRR genes, were up regulated in different cultivars upon *P. sojae* infection. In the latter work, in contrary to the results by Wong et al 2014, miR1507 and miR2109 sequences from the same resistant cultivar (Williams with *Rps1-k*) were reduced upon infection and transcripts of their NBS-LRR target genes were increased. In addition, accumulation of phasiRNAs was also detected in *P. sojae* infected tissues [38,39].

Tomato miRNAs are also involved in plant immunity against *Phytothphora infestans*, the oomycete pathogen that causes the late blight disease. Global profiling of miRNAs response to *P. infestans* has revealed a significant change in 70 miRNAs. Among them, miR6027, miR5300, miR476b, miR159a, miR164a and miRn13 were verified to be reduced after infection, and the corresponding target genes including NBS-LRR (Solyc05g008650.1.1) , *MYB* transcription factor (Solyc01g009070.2.1) , pathogenesis-related transcription factor (Solyc10g076370.1.1) and NAC domain protein (Solyc03g115850.2.1) are all induced [45]. These target genes are plant immunity related, thus proving the concept that tomato miRNAs contribute to plant anti-oomycete defense. In addition, the oomycete pathogen *Pseudoperonospora cubensis* infected cucumber also changes the expression level of 39 known miRNAs, such as miR164b, miR156h, miR171e, miR160b, and miR159f. Consistently, their corresponding target genes are negatively regulated, including Auxin response factor *ARF16*. The down-regulation of miR160 and up-regulation of *ARF16* indicates the positive role of auxin pathway in cucumber defense against the oomycete pathogen [46]. This is opposite to the role of miR160 in anti-bacterial responses, implicating that plant miRNA may act as both positive and negative regulators of defense depending on the nature of the invading pathogen.

In anti-fungi pathogen responses.

Since fungal pathogens cause serious diseases and large economic loss to crops, the role of sRNAs in plant immunity against fungal pathogen attracted more attention recently. This section will focus on the role of sRNAs from different plant species against fungal pathogens.

Wheat is the most grown crop worldwide [47], yet it easily gets infected by various pathogens especially fungal pathogens. The role of wheat miRNAs has been extensively studied against various fungal pathogens including: *Blumeria graminis* causing wheat powdery mildew, *Puccinia graminis* causing wheat stem rust, *Puccinia striiformis* causing wheat strip rust, *Fusarium culmorum* causing foot and root rot and *Fusarium* head blight (FHB), *Bipolaris sorokiniana* causing wheat spot blotch, root-rot and leaf-spot disease. The infection of *B. graminis* on susceptible and resistant wheat cultivars causes miRNA alternations in both cultivars, including 1) response in the susceptible cultivar that contributed only to basal defense, such as down-regulation of miR2001, miR2006, miR2011 and up-regulation of miR393, miR444, miR827, miR2005 and miR2013; 2) response in the resistant cultivar that only regulates plant immunity at the ETI level, such as down-regulation of miR171 and up-regulation of miR2008 and miR2012; 3) responses in both susceptible and resistant cultivars that involve both wheat basal defense and ETI, such as decrease in miR156, miR159, miR164 and miR396 levels [48]. These results are confirmed in a recent study by microarrays. The wheat powdery mildew disease induction of wheat miR528, miR167 and miR394, and reduction of miR156, miR164, miR171, miR396 and miR160 were further confirmed [49]. The microarray assays also identified 66 responsive wheat miRNAs by *F. culmorum* infection and 21 responsive miRNAs by *Bipolaris sorokiniana* infection. Eight of these miRNAs, athmiR869.1, cre- miR1169-3p, mtr-miR2592s-3p, osa-miR1427, osa-miR319a-3p.2-3p, ptc-miR169b-3p, vvi-miR3624-5p, and miR482e, were responsive to both pathogens [50]. In addition, wheat miR408 contributes to plant immunity against wheat stem rust by negatively regulating a

chemocyanin-like protein gene (*TaCLP1*), a positive regulator of plant defense response to *P. graminis* [51]. Furthermore, wheat PN-2013 miRNA suppresses *Monodehydroascorbate reductase gene* (*TaMDHAR*) which leads to the accumulation of H2O2 and higher expression of several *PR* genes, thus becoming more resistant to *P. striiformis* [52].

As discussed above, tomato miR482 is predicted to regulate multiple CNLs genes [36]. In addition, miR6022 and miR6023 also regulate the tomato LRR domain-containing gene *Cf9*, miR6024 and miR6026 target the CNL gene *Tm2*, and miR6027 also targets the CNL gene *Sw5* [35]. Moreover, miR6024 can target at least one homolog of *I2*, the cleavage of *I2* homologs by miR6024 also triggers production of phasiRNAs [53]. Indeed, miR482f and miRNA5300 are repressed by the *F. oxysporum* infection, targeting four NB domain-containing genes related to plant immunity against *Fusarium* wilt disease [54]. Interestingly, one of miR5300 targets is the *R* gene *tm2*, indicating that multiple different miRNAs probably can regulate the same plant immunity pathway [35,54].

B. cinerea is a necrophic fungal pathogen that causes serious grey mold disease on tomato. By using microarray analysis, it has been shown that tomato miR169 is increased yet miR160 and miR171a are decreased by *B. cinerea* infection. The *cis*-element fungal elicitor (Box-W1) accumulates in the promoter region of miR171 and miR160 further confirming their roles in host-fungal pathogen interactions [55]. Recently, global profiling by next generation deep sequencing identified *B. cinerea* responsive tomato miRNAs. 41 miRNA were up-regulated, including miR159, miR169, miR319, miR394, miR1919, and miR1446, whereas 16 were down-regulated, including miR2111, miR5300 and miR160

[56]. As miR5300 is also suppressed upon *F. oxysporum* infection, the same defense pathway can be used by tomato for defense against different type of fungal pathogens [54,56].

In rice, the miRNAs that involved in defense responses against the blast fungus *Magnaporthe oryzae* have been classified into three categories [57]. These are positive regulators, such as miR160a, miR164a, and miR168a; negative regulators, such as miR396, miR827, and miR1871; and basal response regulators, such as miR169a, miR172a, and miR398b. Furthermore, miR160a and miR398b are confirmed to enhance rice disease resistance against *M. oryzae* [57].

In cotton, many miRNAs are altered genome-wide during root infection by the fungal pathogen *Verticillium dahilae*, including miR482, miR472, miR160, miR319, miR399, and miR395 [58]. Interestingly, miR319 and miR395 are reduced in verticillium wilt-susceptible cotton *Gossypium hirsutum* but induced in the wilt-tolerant cotton *Gossypium barbadense*, indicating that the same miRNA may act contrarily in different species [58]. Down-regulation of miR482 in *V. dahilae* infected *G. hirsutum* was confirmed recently. Similar to the role of miR482 in other plant species [34-36,59], cotton miR482 also mediates gene silencing of 36 NBS-LRR genes and activates the processing of phasiRNAs, which probably strengthen the silencing effect of additional *R* genes [60].

In barley, miR9863a and miR9863b regulate a subset of *Mla1* alleles, which encode CNL type R proteins that direct race-specific plant defense responses against the powdery mildew fungus *B. graminis*. The cleavage of *Mla1* also triggers accumulation of phasiRNA

around the cleavage site. Overexpression of miR9863a and miR9863b specifically reduce MLA1-triggered disease resistance and cell death [61]. Moreover, *Mla1* and *Mla6* negatively regulate barley miR398, which guide silencing of the target gene *SOD1* (*Chloroplast copper/zinc superoxide dismutase 1*), thus impairing *Mla*-triggered H2O2 and hypersensitive reaction (HR). The fact that a target gene that is controlled by a miRNA can regulate another miRNA, suggests the existence of a highly complicated gene regulation networks [62].

In maize, miR829, miR845 and miR811 are induced yet miR408 is suppressed during the infection of the fungal pathogen *Exserohilum turcicum*, which causes Northern leaf blight. Overexpression of these miRNAs in maize confirmed the role of miR829 and miR811 in enhancing maize tolerance against this pathogen [63]. In *Arabidopsis*, miR168 and the heterochromatic siRNA siR415 silences *AtAGO1* and *Chromomethylase3* (*AtCMT3*), respectively. Both miR168 and siR415 are transcriptionally activated by the infection of the fungal pathogen *F. oxysporum*, suggesting their positive roles in plant immunity [64]. Since the target of siR415 is involved in RNA-directed DNA methylation (RdDM), the role of RdDM in plant defense responses against fungal pathogen has been evaluated [64,65]. In *Populus,* several miRNAs react similarly to the infection of two different fungal pathogen *Dothiorella gregaria* and *Botryosphaeria dothidea* [66,67], such as miR159, miR164, miR168, miR172, miR319, miR408, miR398, and miR1450; whilemiR160 acted differentially [66,67]. The pathogen related cis-element such as TC-rich repeat, W1-box, and MBS are abundantly present in the promoter region of *populus* fungi-responsive miRNAs also illustrates the function of these miRNAs in plant immunity

[68-71]. In potato, all the family members of miR482 can target a class of NBS-LRR genes, particularly the CNL genes, and trigger secondary siRNAs production. Potato miR482e is down regulated when infected by the fungal pathogen *V. dahilae* leading to the induction of the targeted NBS-LRR genes. The overexpression of potato miR482 also improved immunity against verticillium-wilt disease [59]. In *V. dahilae* infected eggplants, miR393 is significantly reduced and its target *TIR1* is induced, which is in contrast to the *Arabidopsis* miR393 in response to bacterial pathogen. In eggplant, inhibition of the auxin pathway increased verticillium wilt disease. Additional conserved miRNAs in eggplant, including miR399, miR395, miR171, miR164, miR172 are also involved in immunity to *V. dahilae* [72].

In fact, many additional plant species have been found that use sRNAs to inactivate or activate genes involved in plant immunity during fungal pathogen infections. For example, oilseed rape against *V. dahilae* [73], cassava against anthracnose disease fungal pathogen *Colletotrichum gloeosporioides* [74], Norway spruce against blue stain fungal pathogen *Ceratocystis polonica* [75], and the model grass species *Brachypodium distachyon* against the fungal pathogen *F. culmorum* [76].

The role of filamentous pathogens sRNAs in host-pathogen interactions.

In this section, I will describe the function of pathogen sRNAs in two aspects: (i) pathogen endogenous sRNAs that regulate important virulence genes (effectors) during infection within pathogen cells, and (ii) pathogen sRNAs that translocate from the pathogens into the host plant cells during infection to silence host immunity genes. These

pathogen-produced sRNAs, which direct silencing of host immunity genes, are termed sRNA effectors. Host gene silencing by pathogen sRNA effectors describes a new chapter of cross kingdom RNAi events during host–pathogen interactions.

Pathogen sRNAs regulate effector genes within pathogen cells to achieve virulence.

Many pathogens produce effectors to suppress host plant immunity as part of their virulence strategy. Two of the best-characterized eukaryotic effector classes are RxLR motif-containing effectors and Crinkler (CRN)-type effectors [77-79]. Both classes of effectors are commonly known from the oomycete plant-pathogenic *Phytophthora* spp. Host plants of *Phytophthora* evolve *R* gene-based resistance, which recognizes RxLR and CRN effectors, and triggers ETI [5,7].

Phytophthora infestans is the causal agent of late blight and of the disastrous potato famine in the 18th century in Ireland. *P. infestans* is expected to produce hundreds of protein effectors during infection. In total, more than 500 RxLR and over 300 CRN effector genes have been predicted in the *P. infestans'* genome. However, only a few of these putative effectors have been proven to be essential for pathogenicity, which is probably a result of combinatorial effects, host-specific activity and redundant functionality. Remarkably, tight spatial–temporal regulation of effector expression occurs [78,79].

Recently, genome-wide transcriptomic studies have revealed that *Phytophthora* produces masses of sRNAs that map to genomic regions of RxLR and CRN genes. This observation suggests that expression of these effector genes is controlled by regulatory sRNAs. Indeed, accumulation of sRNAs has been shown to correlate with

silencing of these effector genes. Remarkably, sRNA populations are distinct among different phytopathogenic *Phytophthora* spp. [80]. Moreover, significant differences in sRNAs, which map to effector gene sites, have been revealed between two *P. infestans* isolates that show different virulence levels on the host potato [81]. We are awaiting a more detailed study on the relationship between sRNA accumulation intensities at effector gene sites and the virulence performance of different *Phytophthora* strains.

The soybean pathogen *Phytophthora sojae* is a close relative of *P. infestans*. Qutob *et al*. observed sRNA-mediated silencing of another effector gene, *Ps-Avr3a* [82]. Interestingly, silencing was observed in the *P. sojae* virulent strain ACR10, but not in the avirulent strain P7076 (*Avr3a)* when infecting soybean plants carrying the *R gene Rps3a*. In support of this, the level of sRNAs derived from the *Avr3a* locus was much higher in the ACR10 strain than in the avirulent P7076. Here, unlike the usual positive role of effectors in host plant infection, silencing of an effector gene seems to be of advantage to the pathogen. Under the described circumstances, keeping an effector gene silenced might help avoid its detection by the corresponding host R protein to escape host immune responses and achieve compatibility. This shows that a *P. sojae* strains have evolved such an adaptive strategy to bypass *R* gene-mediated resistance in host plants. By silencing of an effector, the host ETI trigger, the ACR10 strain is able to infect its host plant without triggering a fatal resistance. The reversible silencing of an effector gene by sRNAs is assumed to be more advantageous than the irreversible loss of effector function by a gene mutation, because the re-activation of a silenced effector might strengthen virulence when its producer infects a new host plant that lacks the corresponding *R* gene to this effector.

Phytophthora effectors often reside in TE-rich regions, which give rise to many sRNAs [83,84]. The fine-tuned expression patterns of these effectors during infection are possibly regulated by sRNAs in order to adapt to various host plants. However, we are still at the beginning of our understanding of how effector gene expression is controlled and what are the underlying mechanisms. sRNAs act through RNAi machinery and guide gene silencing, and the RNAi pathway components are indeed functional in *Phytophthora*. Fungal RNAi pathways are very diverse and complex, with only a subgroup of sRNAs being DCL dependent [85,86]. Similarly, only a subgroup of sRNAs from *Phytophthora* is dependent on DCLs. The sRNAs that map to effector gene loci are mostly DCL1 dependent and probably regulate the expression of effector genes.

Transcriptional control via sRNA-guided DNA methylation has been observed in animal and plant species, predominantly in TE-rich regions. Local spreading of DNA methylation patterns from TEs to nearby protein-coding genes has been described. Although sRNA-directed DNA methylation has not been observed in fungal or oomycete systems, epigenetic control, such as histone modification, has been proposed to regulate gene-silencing pathway in *P. infestans* as silencing of a sporulation-associated gene was found to require a histone deacetylase [87].

Similar genomic organization of effector genes in TE-rich regions has been found in other notorious fungal plant pathogens, such as *Blumeria* and *Leptosphaeria* [88]. In *Leptosphaeria maculans,* epigenetic control of effector genes is linked to heterochromatin formation via methylation of the histone H3 lysine 9 [89]. Many effector genes are activated during infection, some possibly through epigenetic activation.

The extent and conservation of the regulation of expression of effectors or other virulence factors by sRNAs among diverse pathogens remains to be clarified. Silencing of effectors to avoid ETI might be a special virulence strategy that has evolved in *Phytophthora*. Activation of effectors, which are host immunity suppressors and infection facilitators, is expected to be more common during infection. Indeed, several sRNAs have been found in the rice blast pathogen *Magnaporthe oryzae* which have been predicted to target virulence-related genes, among them the avirulence gene *ACE1*. Expression of *ACE1* was de-repressed in RNAi mutants of *M. oryzae*, probably as a result of blocking of the production of regulatory sRNAs [88]. Expression of *ACE1* is strictly controlled and is induced only during appressoria formation, a specialized cell formation for initial penetration into plant tissues. It is likely that sRNAs silence *ACE1* under non-infectious conditions, whereas sRNAs are switched off at local sites of host infection in order to activate *ACE1* expression. We speculate that pathogen sRNAs that suppress virulence genes under non-infectious conditions and during saprophytic growth are very common. For infection, expression of such sRNAs might be switched off leading tos activation of virulence genes.

Pathogen sRNAs are delivered into host cells and act as effectors to suppress host immunity.

Pathogen effectors are molecules that are delivered into host cells to suppress host immunity. Most effectors that have been studied so far are proteins. A recent study has assigned a similar behavior to *B. cinerea* sRNAs (Bc-sRNAs), which are non-proteinaceous effectors in its virulence arsenal. *Botrytis cinerea* is an aggressive pathogen

with a broad host range, which can infect more than 200 different plant species. Bc-sRNAs are transported into host cells during infection and silence important plant immunity genes, as shown in two hosts, *Arabidopsis* and tomato. In total, more than 70 Bc-sRNAs have been identified to be potential effectors based on *in planta* expression and target gene prediction in both *Arabidopsis* and tomato hosts, for which three sRNA effectors have been demonstrated experimentally to silence host plant immunity genes by hijacking host RNAi machinery [24]. Silencing of host immune genes ensures successful infection of *B. cinerea* in host plants [24]. These Bc-sRNA effectors share common features with host sRNAs that are favorably sorted into *Arabidopsis* AGO1 (AtAGO1) protein, and thus utilize the host RNAi machinery by loading into host AGO1 to silence host immunity genes. In support of this, the *Arabidopsis* mutant *ago1-27* was less susceptible to *B. cinerea*, because the Bc-sRNA effectors were no longer functional in guiding the host gene silencing without the appropriate AGO protein [24].

This is the first report of pathogen sRNAs acting as effectors to inhibit host immunity. Future research will unveil whether this novel sRNA-based virulence pathway also exists in other plant eukaryotic pathogens. Indeed, another aggressive fungal pathogen, *V. dahilae*, may have evolved a similar strategy of hijacking the host plant RNAi machinery to suppress host immunity. Similar to that observed during *B. cinerea* infection, the *Arabidopsis ago1-27* mutant was more resistant against *Verticillium* spp., whereas several other *Arabidopsis* RNAi mutants exhibited enhanced susceptibility [90]. Thus, Arabidopsis AGO1 is also required for *V. dahilae* pathogenicity.

Long terminal repeat (LTR) retrotransposons produce mass of sRNAs that provide a large selective pool of sRNA regulators for pathogenicity

TEs are mobile genomic elements that drive genome evolution. TE replication and transposition are associated with genomic DNA rearrangements and mutations. Although temporal transposition activity has beneficial effects in terms of adaptive evolution, it is obvious that such elements can be detrimental. The class of LTR retrotransposons is widespread among eukaryotes [91-93]. LTRs proliferate by transcription of an RNA intermediate that is reversely transcribed into complementary DNA and subsequently re-integrates into the host genome by random insertion. LTR regions are hot spots of sRNA production. LTR RNA intermediates probably serve as templates for RNA-dependent RNA polymerases that synthesize a complementary RNA strand. Double-stranded RNAs are processed by DCLs to produce masses of sRNA molecules. The primary function of these sRNAs within fungal pathogens is to silence LTRs to maintain genome integrity.

Protein effector genes are often clustered and located in TE-enriched chromosomal regions, where housekeeping genes are largely depleted. For instance, RxLR and CRN effectors of *Phytophthora* spp. are often located in close vicinity to LTRs. The spread of transcriptional silencing from LTR loci onto nearby coding genes has been found in other eukaryotes. Indeed, RxLR and CRN genes are often found to be within a distance of 2 kb of LTRs in *P. infestans*, which represents an evolutionary advantage for the fast turnover of effectors [81,94]. The majority of Bc-sRNAs predicted to silence host plant genes are also derived from a class of LTRs in *B. cinerea*, the so-called Boty-like elements. Such gene arrangement suggests that Boty LTRs possibly play a positive role in driving the fast

evolution of Bc-sRNA effectors in *Botrytis*. This might lead to the rapid adaptation of *Botrytis* to a wide range of host plants, rendering this fungal pathogen into a highly aggressive, broad-spectrum pathogen. The temporal activation of TEs under stress has been observed in different organisms. Likewise, transcriptional expression of LTRs is strongly induced in various eukaryotic pathogens, such as *P. infestans*, during sporulation, germination and appressoria formation. Apparently, the induction of LTRs results in greater accumulation of LTR-associated sRNAs, which not only control LTR expression, but also provide a large pool of sRNAs for selection of effectors towards different hosts. In certain cases, LTR-derived sRNAs can silence neighbor protein effector genes, which may also be an adaptive strategy during infection to escape ETI, as discussed above.

Interestingly, Boty elements genetically associate with virulence and host preference in *B. cinerea*. Population genetics studies have revealed that *B. cinerea* field isolates collected from geographically diverse and independent locations show a domination of Boty-carrying isolates (called transposa) in areas of massive crop (host plant) production. Transposa isolates are significantly more virulent than others. Bc-sRNA effectors physically link to Boty elements and may facilitate the fast turnover of Bc-sRNAs, which would be of evolutionary advantage for the pathogen during the molecular arms race against host plants [19].

Cross-Kingdom RNAi in Host Plant–Pathogen Interaction

Cross-kingdom RNAi describes the phenomenon in which a donor organism produces an RNAi trigger that moves into a recipient organism and causes gene silencing.

Cross-kingdom RNAi occurs during host plant–pathogen interaction, and can take place in both directions: (i) sRNAs produced by a pathogen to be delivered into host cells to silence host genes; and (ii) a host-produced gene silencing trigger to suppress pathogen gene(s). The sRNA effectors that are produced by *B. cinerea* translocate into host cells to silence plant immunity genes. Host-induced gene silencing (HIGS) studies have shown that a transgenic silencing trigger is expressed in plants, which then translocates into infecting pathogen cells to turn down virulence gene expression. HIGS is a well-established molecular tool to achieve plant resistance against various pathogens and pests.

HIGS is based on an artificially designed RNAi trigger against pathogen virulence genes. We speculate that the export 'channel' for the RNAi trigger is not only prepared for artificial transgenic sRNAs, but that some host endogenous RNAi triggers or sRNAs are also transported into certain pathogen cells for gene regulation. This is quite likely because cross-kingdom RNAi has been described in diverse biological systems. For instance, sRNAs from plants consumed as food have been detected in human and animal serum [95]. HIGS is effective in diverse plant species and against different pathogens and pests, indicating that the basic cellular inventory required for cross-kingdom RNAi seems to exist ubiquitously in plants, animals and filamentous microbes [96-98]. Thus, the identification of a natural plant-produced gene-silencing trigger has great potential as a novel molecular marker in host resistance against pathogens and pests.

Cross-kingdom RNAi events demonstrate that gene silencing signals can travel extracellularly over long distances and, in terms of plant–microbe interaction, across plant and pathogen cell walls, membranes, cuticular layers and other cellular boundaries.

However, the underlying mechanisms of trafficking of RNAi signals still remain enigmatic. For example, the application of HIGS is successful in *Phytophthora capsici* [99], but does not seem to work efficiently in a related species *Phytophthora parasitica* [100]. Although more experiments on other HIGS-targeting genes are needed to confirm this observation, an understanding of how the RNAi signals travel between hosts and pathogens/pests is a major task in the field, and will help to address this question. In addition, another open question concerns what form and nature of mobile gene silencing signals exist in cross-kingdom RNAi: single-stranded sRNAs, double-stranded sRNAs or long double-stranded sRNA precursors? Systemic RNA gene silencing has been shown in plants and animals. In plants, mature sRNAs can spread from cell to cell at approximately 10–15 adjacent cells from the origin of production, most probably via plasmodesmata [101,102]. RNAi signals can also move systemically over long distances via the phloem to mediate gene silencing [103-105]. In contrast, systemic RNAi in *Caenorhabditis elegans* is associated with longer RNA molecules, the precursors of mature sRNAs [106]. S*ystemic RNAi-deficient* (*SID*) genes have been identified to be required for the cellular uptake of environmental RNA and cell-to-cell RNA transport [107-109]. Interestingly, *SID* genes have been exclusively found in invertebrates, but not in plants, oomycetes or fungi, indicating a unique pathway of environmental and systemic RNAi in invertebrates.

The characterized Bc-sRNA effectors possibly translocate as sRNA duplexes or mature sRNAs, rather than longer RNA precursors, and load directly into the plant AGO protein to silence host immunity genes. Infection assays on *Arabidopsis dcl1* (*Atdcl1*) mutants with *B. cinerea* revealed an enhanced susceptibility phenotype, which indicates

that Bc-sRNA-induced host gene silencing was not disturbed in the *Atdcl1* mutant, and the host RNAi pathway may contribute to plant natural defense against *B. cinerea*. Moreover, *B. cinerea dcl1/dcl2* mutant was unable to produce Bc-sRNA effectors, and consequently failed to suppress host immunity genes during infection, thus exhibiting a weakened virulence phenotype compared with the *B. cinerea* wild-type [24]. It would be worthwhile to determine whether other eukaryotic pathogens could also utilize similar strategies to deliver sRNA effectors into host cells to trigger silencing of host plant immunity genes.

Future research is needed to elucidate what are the underlying molecular mechanisms of RNA export from an infecting pathogen cell and the uptake into the host plant cells. How do sRNAs move across diverse cellular boundaries? Is this process based on an active specific transport ‘channel'? It seems that there is a selective process for choosing Bc-sRNAs to be delivered into host cells, because not all Bc-sRNAs are found in host cells. What is the selection mechanism? Softening of the plant cell wall and membrane by pathogen-secreted degrading enzymes might ease the entrance of sRNA effectors into host cells during the infection process. Another fundamental yet basic question is what protect cross-kingdom sRNAs from degradation in the extracellular matrix. In mammals, extracellular sRNAs are often associated with RNA-protective protein complexes and/or encapsulated into extracellular vesicles [110]. Do such protective proteins and vesicles also exist for the transport of sRNAs between plants and microbes?

The discovery of pathogen RNA effectors that suppress host immunity has increased our understanding of the molecular arms race between pathogens and host plants. sRNA-triggered interspecies gene silencing seems to be an additional regulatory layer for host–

pathogen interaction. From the evolutionary point of view, the physical contact of pathogen RNA effectors with host cellular components must enforce the evolution of a counter-defense strategy to defeat RNA attack. Normally, host plant receptor proteins recognize conserved PAMPs or pathogen protein effectors, and induce a host immune reaction. Are microbial RNA molecules recognized by receptor molecules directly or indirectly to stimulate defense responses? The receptor proteins that recognize PAMPs or effectors and initiate immune responses in animals usually belong to the class of TLRs. TLRs are described as resistance factors, which can also recognize conserved pathogen DNA elements to stimulate immunity. Interestingly, a recent report has claimed that a bacterial pathogen-derived ribosomal RNA molecule activated TLR signalling and induced an immune response in mice [111] In addition, it has been demonstrated that endogenous extracellular sRNAs, such as microRNAs (miRNAs), activate membrane-associated TLR receptors for immune reaction in human natural killer cells [112]. It would be worthwhile to determine whether plants have evolved similar receptors that recognize microbial RNA molecules to trigger innate immune responses against microbial attackers. In this context, we speculate that extracellular RNA molecules might be multifunctional in host–pathogen interactions. In particular, cell-non-autonomous sRNAs might be a *lingua franca* in interspecies RNAi communication affairs.

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Chapter 2

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

Abstract

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 dcl1 dcl2* 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.

Introduction

Botrytis 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 and completed whole genome sequence, *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.

Results

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. lycopersicumlibraries* 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 2.2). 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 (Figure 1.1, A and B; Figure 1.2; and table 1.2) and were the major sRNA products from their encoding loci, LTR retrotransposons (Figure 1.2). 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* (*PRXIIF*), which is targeted by Bc-siR3.1; and *cell wall-associated kinase* (*WAK*), which is targeted by Bc-siR5 (Figure

1.1C). 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 (Figure 1.1C). 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 (Figure 1.1B) 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 (Figure 1.1D).

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 (Figure 1.1E). The silencing was abolished, however, when the target genes carried a synonymously mutated version of the relevant Bc-sRNA target sites (Figure 1.1E and Figure 1.3A). We also observed suppression of yellow fluorescent protein (YFP)–tagged target *MPK2* by *B. cinerea* infection at 24 hours after inoculation (Figure 1.1F and Figure 1.3B); when the Bc-siR3.2 target site of *MPK2* was mutated, infection by *B. cinerea* failed to suppress its expression (Figure 1.1F and Figure 1.3B). 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 (Figure 1.3C), only the wild-type sensor was suppressed after *B. cinerea* infection (Figure 1.1G).

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 (Figure 1.4A) [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 (Figure 1.4B). With pathogen challenge, all of the Bc-sRNAox lines displayed enhanced susceptibility to *B. cinerea* (Figure 1.4, 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* (Figure 1.4, D and E). A transferred-DNA knockout mutant of the Bc-siR5 target *WAK* (SALK_089827) (Figure 1.5A) also displayed enhanced susceptibility to *B. cinerea* (Figure 1.4, 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* (Figure 1.5B). 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 rattle virus (TRV) (Figure 1.6A)[19]. VIGS of TRV-*MAPKKK4* caused a dwarf phenotype (Figure 1.6B). 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 (Figure 1.4F). 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 1.3). This sRNA structure is favored for binding to AGO1 in *Arabidopsis* [20,21]. In order to determine whether Bc-sRNAs act through *Arabidopsis*, 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 (Figure 1.7A) or in the AGO2- and AGO4-associated sRNA fractions (Figure 1.8). 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 (Figure 1.9).

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 (Figure 1.7B and Figure 1.10A). Consistent with this, *BIK1*

induction was increased compared with that of the wild-type (Figure 1.10B). Furthermore, the expression of Bc-siR3.2 targets *MPK2* and *MPK1*, Bc-siR3.1 target *PRXIIF*, and Bc-siR5 target *WAK* in *ago1-27* was not suppressed compared with those in wild-type infected plants after *B. cinerea* infection (Figure 1.7C). 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* (Figure 1.7D). These results suggest that the increased resistance phenotype we observed in *ago1-27* is not caused by any reduced vigor 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 DCL* genes, which encode the core sRNA processing enzymes. *B. cinerea* strain B05.10 possesses two *Dicer-like* genes (*Bc-DCL1* and *Bc-DCL2*) (Figure 1.11). We generated *dcl1* and *dcl2* single and *dcl1 dcl2* double knockout mutant strains through homologous recombination (Figure 1.12, A and B). We found that *dcl1* and *dcl2* single mutants showed reduced growth and delayed sporulation (Figure 1.12C). 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 (Figure 1.13A), indicating that they were DCL-dependent, whereas two other Bc-sRNAs, Bc-milR2 and Bc-siR1498, could still be detected in *dcl1 dcl2* double mutant (Figure 1.12D). 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 (Figure 1.13, 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* (Figure 1.14), 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 (Figure 1.12C) but had no effect on pathogenicity (Figure 1.13, B and C). Furthermore, expression of the YFP sensor carrying the Bc-siR3.2 target site in *N. benthamiana* was silenced when infected with wild-type *B. cinerea*. The suppression was abolished when inoculated with the *dcl1 dcl2* strain (Figure 1.13D), 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 tomato upon infection (Figure 1.13E). Consistent with this, the *dcl1 dcl2* virulence was partially restored when infected on *Arabidopsis* Bc-siR3.1ox and Bc-siR3.2ox plants as well as in tomato TRV-*MAPKKK4*–silenced plants (Figure 1.13, F and G).

Discussion

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, *Verticllium 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.

Materials and Methods

Generate *dcl1*, *dcl2* single and double mutants of *B. cinerea*

By using homologous recombination and the *Agrobacterium tumefaciens*-mediated transformation system adapted from Utermark and Karlovsky [29], we generated *dcl1*, *dcl2* and *dcl1 dcl2* deletion mutants in *B. cinerea* strain B05.10. Transformants were selected with 70 ppm hygromycin or 100 ppm NH^4-glufosinate.

Plant materials and protocols

Plant materials used in this study are: *Arabidopsis thaliana* ecotype Col-0, *Solanum lycopersicum* (tomato) cultivar Moneymaker, and *Nicotiana benthamiana*, *Arabidopsis* knockout mutants *mpk1 mpk2* (SALK_063847xSALK_019507) [18] and *wak* (SALK_089827).

The Gateway pEarley vectors (with YFP & HA tags) were used for expression of BcsRNA target genes [30]. Bc-sRNAs were cloned into the miRNA319a backbone vector [17] and transferred into the Gateway vector pEarley100 (without tag) for expression. Transient co-expression assays in *N. benthamiana* were performed as described in[8]. Virus-induced gene silencing (VIGS) was performed by cloning a 294-bp *MPKKK4* gene fragment into the TRV2 vector [19].

Pathogen assay

Four-week-old plants were inoculated by applying a single 20 µl droplet per leaf or by spray-inoculating the entire plant, using $2x10^5$ spores /ml for *Arabidopsis* and $1x10^4$ spores/ml for *S. lycopersicum* and *N. benthamiana.* Disease was assessed by measuring lesion size (ImageJ software) and/or by quantifying *B. cinerea* biomass using quantitative PCR with *B. cinerea*-specific ITS primers (Figure S4).

Confocal microscopy

YFP-tagged protein expression in *N. benthamiana* was quantified using the confocal microscopy system Leica SP2. Z-series images (10 images in a distance of 0.7µM) were

merged to gain average signal intensity. Merged images were exported as TIFF files and YFPquantity was measured using the ImageJ software.

AGO immunoprecipitation (IP)

Arabidopsis AGO IP [8] was conducted with 5 g fresh leaves collected at 24, 32 and 48 h after spray inoculation with *B. cinerea*. Uninfected leaves mixed with at least double amount of *B. cinerea* biomass as in 48 hpi samples were used as a control. AGO1 was purified with a peptide-specific antibody. AGO2 and AGO4 IPs were conducted using native promoter-driven transgenic epitope HA-tagged and c-MYC-tagged lines, respectively and commercial HA and c-MYC antibodies.

sRNA RT-PCR

RNA was extracted from *B. cinerea*-infected plant tissue or the AGO pull-down fraction using the Trizol method. Purified RNA was treated with DNase I and then used in RT-PCR [31] to detect Bc-sRNAs. 35-40 cycles were used for detecting Bc-sRNAs, 22-28 cycles were used for detecting actin genes from *Arabidopsis*, *S. lycopersicum* and *B. cinerea*. Primers used for reverse transcription and amplification of Bc-siRNAs are listed in Table 1.4.

sRNA cloning and Illumina HiSeq data analysis

sRNAs (18-28 nucleotides) were isolated by 15% PAGE and libraries were constructed using the miRCat cloning system and deep sequencing was performed on an Illumina HiSeq 2000. The sequence datasets of sRNA libraries from *B. cinerea*

(GSE45320), *B. cinerea*-infected *Arabidopsis* (GSE45323) and *B. cinerea*-infected *S. lycopersicum* (GSE45321) are available at the NCBI database. The sRNA sequencing reads were preprocessed with the procedure of quality control and adapter trimming by using fastxtoolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Following adapter trimming, sequences were mapped to *B. cinerea* B05.10, *Arabidopsis* (TAIR10), or S. lycopersicum (ITAG_SL2.40) genomes and only the reads that matched perfectly to each genome were used for further analysis. The read number for each distinct sRNA was normalized to the total *B. cinerea* mapped reads in *B. cinerea*-infected *Arabidopsis* and *S. lycopersicum* libraries. The ratio of total *B. cinerea* mapped reads of *Arabidopsis* and *S. lycopersicum* libraries is 2.5:1, so we divide the normalized siRNA read number of *S. lycopersicum* by 2.5.

The sRNAs we selected have satisfied the following conditions: 1) it must be present in both *B. cinerea*-infected *Arabidopsis* and *S. lycopersicum* libraries; 2) its normalized read number was larger than 100 in *Arabidopsis* or *S. lycopersicum* libraries; 3) its normalized reads must be higher than that in cultured *B. cinerea* libraries and 4) it has predicted targets in both *Arabidopsis* and *S. lycopersicum*.

Target gene prediction for Bc-sRNA was performed using TAPIR1.1 [32] with more stringent requirement than described in [32]. No gap or bulge within the alignment between the sRNA and the target was allowed, and the 10th nucleotide of the sRNA must perfectly match its target. At most one mismatch or two wobbles was allowed from position 2 to 12. A maximum of two continuous mismatches was allowed and a score of 4.5 was used as a cutoff.

If a sRNA has predicted targets in both *Arabidopsis* and *S. lycopersicum*, it was selected. The sRNAs were grouped if their 5' end position and 3' end position were within 3 nucleotides on the genomic loci. We presented the selected sRNAs with targets in both Arabidopsis and *S. lycopersicum* in table S3.

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Figures and Tables

Figure 1.1 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. Similar results were seen in three biological 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 *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).

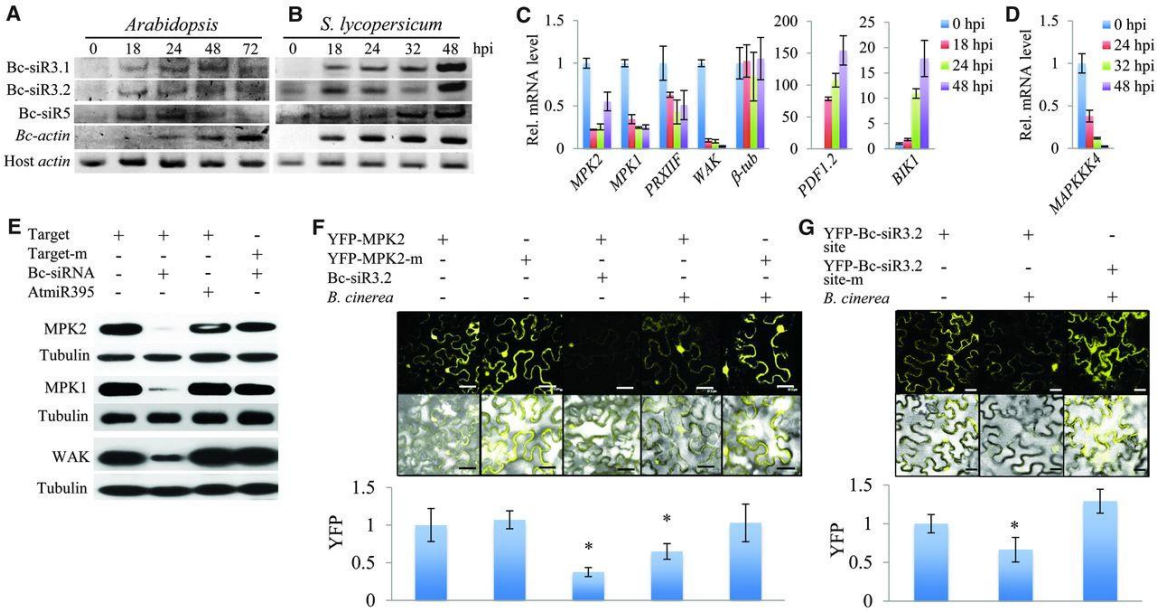

Figure 1.2 Genomic map and read distribution of Bc-SIR3 and Bc-SIR5 loci

The genomic regions of 60 nt up- and downstream of the Bc-sRNA of interest were included. Sequence reads of Bc-siR3 and Bc-siR5 in *B. cinerea*-infected *Arabidopsis* (0, 24, 48, 72 hpi), *B. cinerea*-infected *S. lycopersicum* (leaf/fruit 0, 24, 72 hpi), or in vitro culture *B. cinerea* sRNA libraries (conidiospores, mycelia, total biomass) (see table 1.1) are shown in three individual panels. Bc-siR3 and Bc-siR5 reads are in red. In vitro culture *B. cinerea* sRNA libraries did not show a clear peak for Bc-siR3.1 or Bc-siR3.2 compared to *B. cinerea*-infected *Arabidopsis* and *S. lycopersicum* libraries, indicating that those Bc-siRNAs were induced during infection. Similarly, Bc-SIR5 showed induction upon infection.

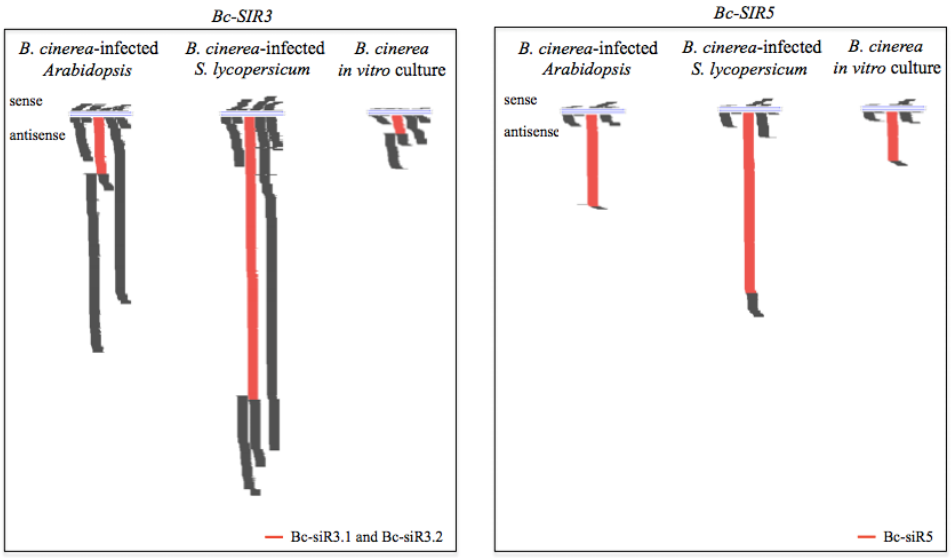

Figure 1.3 Bc-siRNA specifically silence Arabidopsis target genes

A. Target site and target site mutated versions of Bc-siRNA Arabidopsis target genes that were used in this study. B. *B. cinerea* mycelium coincided with target gene suppression of YFP-MPK2 (center), but not YFP-MPK2-m (right) in *N. benthamiana* at 24 hpi; YFP-MPK2 without fungal infection was used as a control (left). Upper panel: YFP; bottom panel: YFP/bright field overlay; scale bar: 50 μm. C. A schematic diagram of the YFP sensor carrying a Bc-siR3.2 target site.

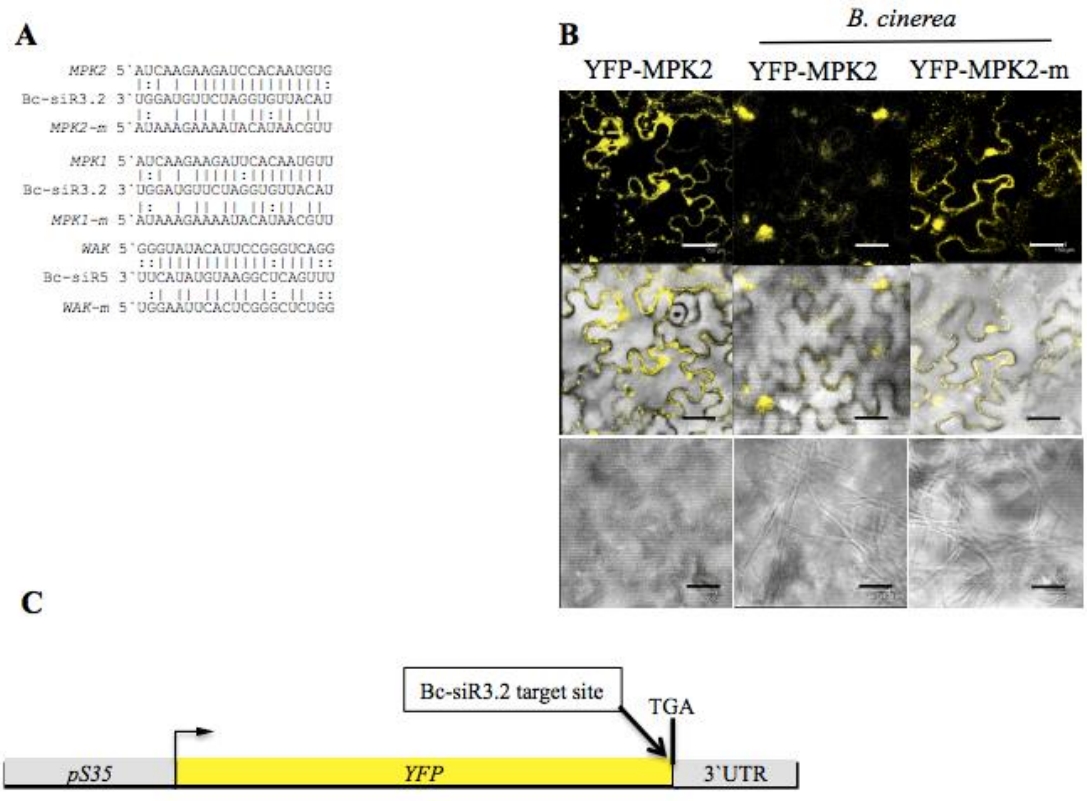

Figure 1.4 Bc-sRNAs trigger silencing of host targets that are involved in host immunity

(**A**) Expression of Bc-siR3.1, BcsiR3.2, 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).

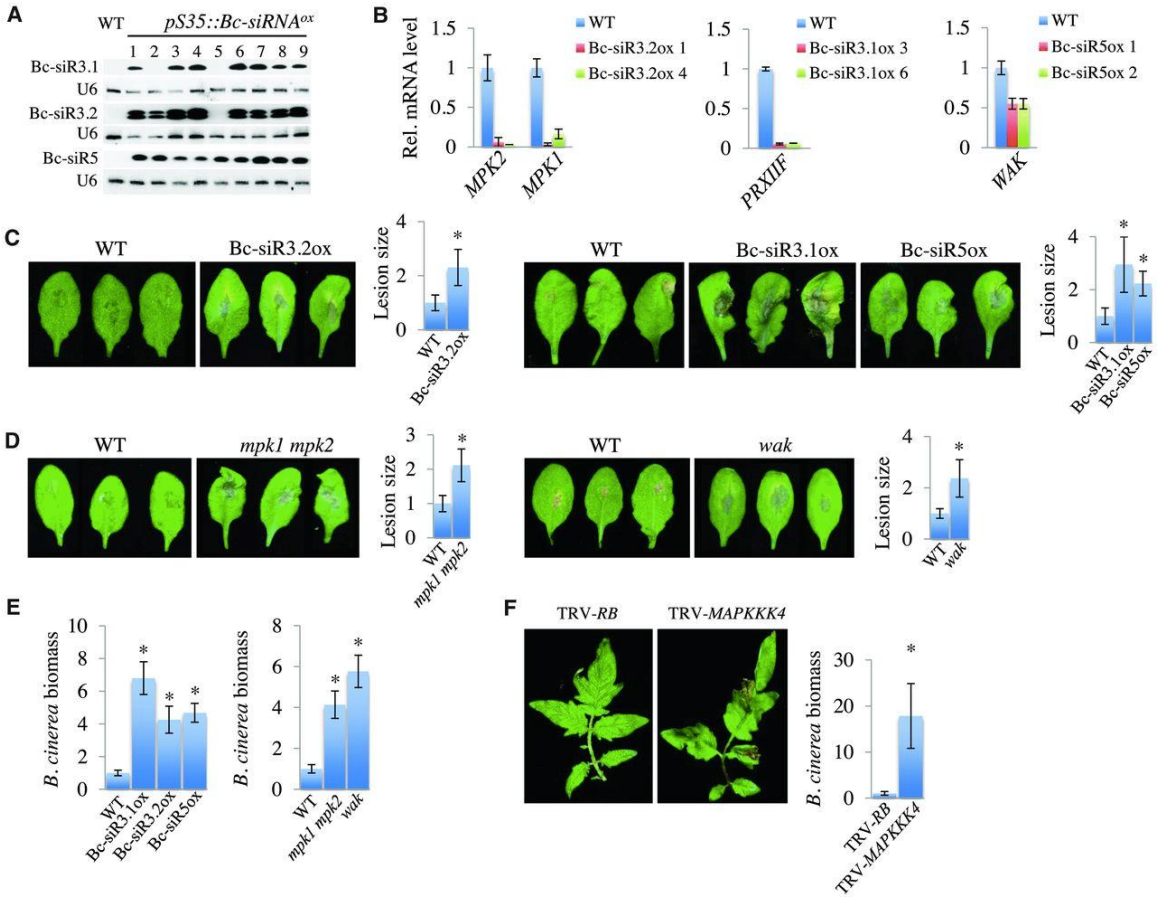

Figure 1.5 Isolation and characterization of Bc-siRNA target mutants and Bc-siRNAox lines

A. Isolation of a loss-of function mutant line for *WAK* gene. Expression of *WAK* was completely knocked out in the T-DNA insertion line shown by RT-PCR. B. Induction of *BIK1* expression in response to *B. cinerea* infection was reduced in Bc-siR3.1ox and Bc-iR3.2ox lines, *mpk1 mpk2*, and *wak* mutant lines. Relative transcript levels of *BIK1* were measured by real time RT-PCR. Error bars indicate standard deviation (SD) of three technical replicates. Similar results were obtained from two biological repeats.

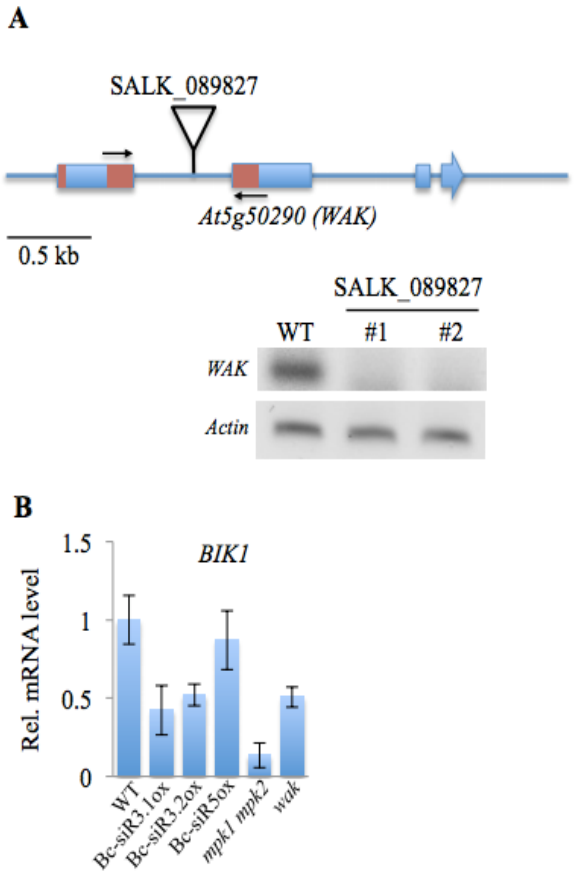

Figure 1.6 *S. lycopersicum MAPKKK4* gene knockdown by TRV-induced gene silencing

A. Expression of *MAPKKK* in S. lycopersicum TRV-*MAPKKK4* silenced plants was measured by qRT-PCR using actin as an internal control. Error bars indicate SD of three technical replicates. Similar results were obtained from three biological repeats. B. TRV*MAPKKK4* silenced plants exhibited a dwarf phenotype as compared with control plants (TRV-RB).

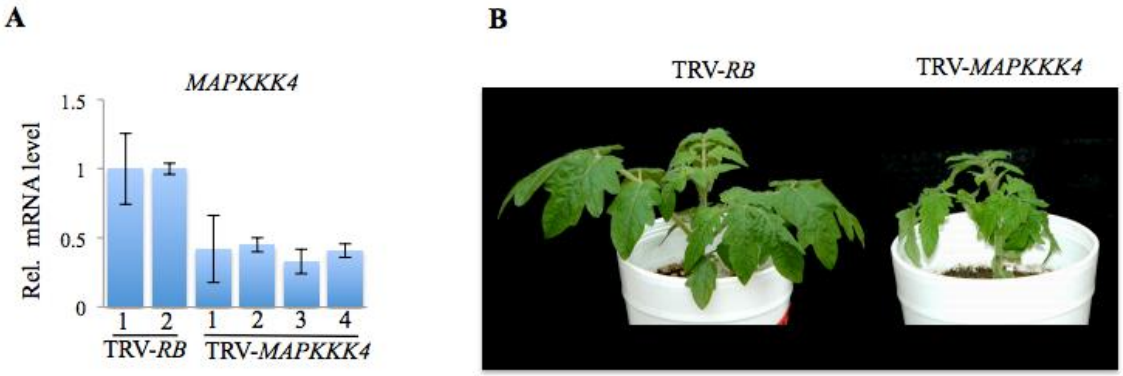

Figure 1.7 Bc-sRNAs hijack *Arabidopsis* AGO1 to suppress host immunity genes

(A) Loading of Bc-siR3.1, Bc-siR3.2, and Bc-siR5 into *Arabidopsis* AGO1 during infection was detected with AGO1-IP followed by RT-PCR. AGO1 from *B. cinerea*–infected leaves harvested at 24, 32, and 48 hours after inoculation was pulled down by AGO1 peptide antibody, and RNA was extracted from the AGO1-IP fraction. As a control, noninfected leaves mixed with*B. cinerea* mycelium (at least twice as much as that in*B. cinerea*–infected leaves at 48 hours after inoculation) 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 hours after inoculation. (**C**) Silencing of *MPK2*, *MPK1*, *PRXIIF*, 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).

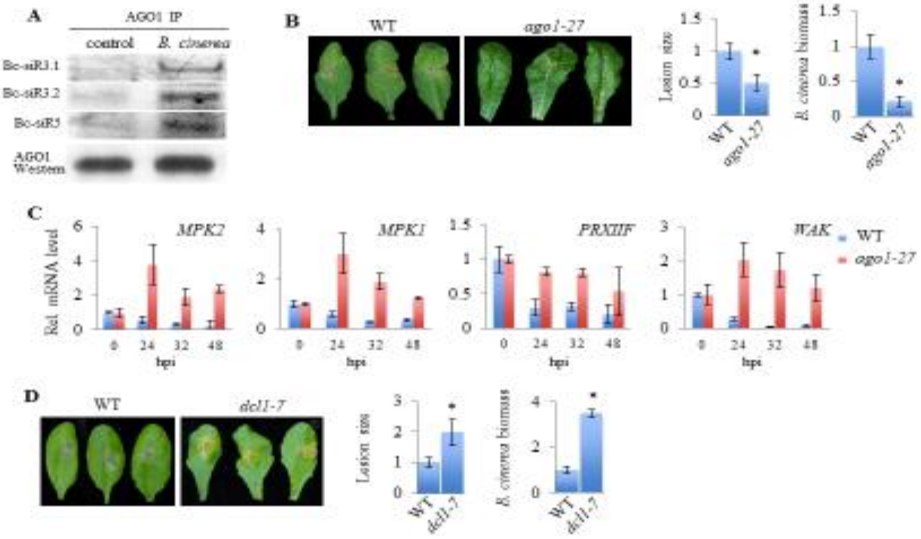
A
AGO1 IP
control
B. cinerea
Bc-siR3.1
Bc-siR3.2
Bc-siR5
AGO1 Western
B
WT
ago1-27
Lesion size
B. cinerea biomass
C
MPK2
MPK1
PRXIIF
WAK
Rel. mRNA level
hpi
WT
ago1-27
D
WT
dcl1-7
Lesion size
B. cinerea biomass

Figure 1.8 Bc-siR3.1 and Bc-siR5 were specifically loaded into *Arabidopsis* AGO1 during infection, but not into AGO2 or AGO4

As revealed by AGO-IP followed by RT-PCR. Endogenous plant sRNAs were used as internal controls for IP: At-miR398a for AGO1, AtmiR393b* for AGO2, and At-siR1003 for AGO4.

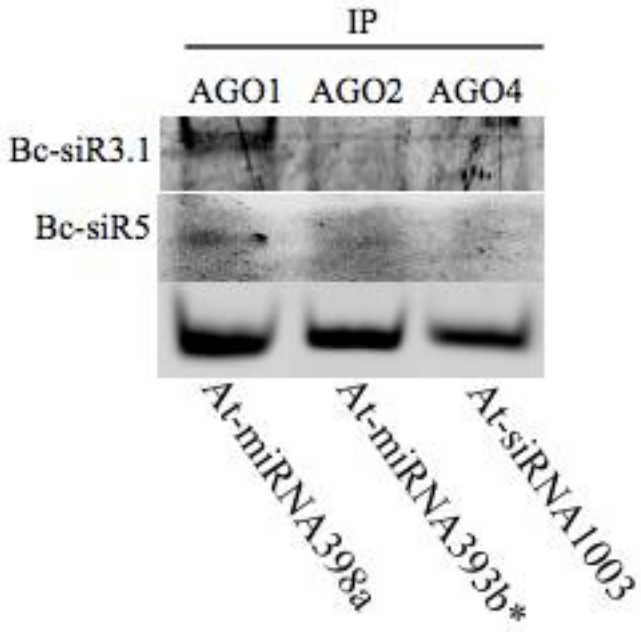

Figure 1.9 sRNA with no predicted plant targets or have predicted targets that were not down-regulated by B. cinerea infection didn't associate with AGO1

The sRNAs that have no predicted plant targets (Bc-siR394, Bc-siR233, Bc-siR269) or have predicted targets that were not down-regulated (Bc-siR9, Bc-siR24, Bc-siR67) by *B. cinerea* infection are not present in the AGO-associated fractions

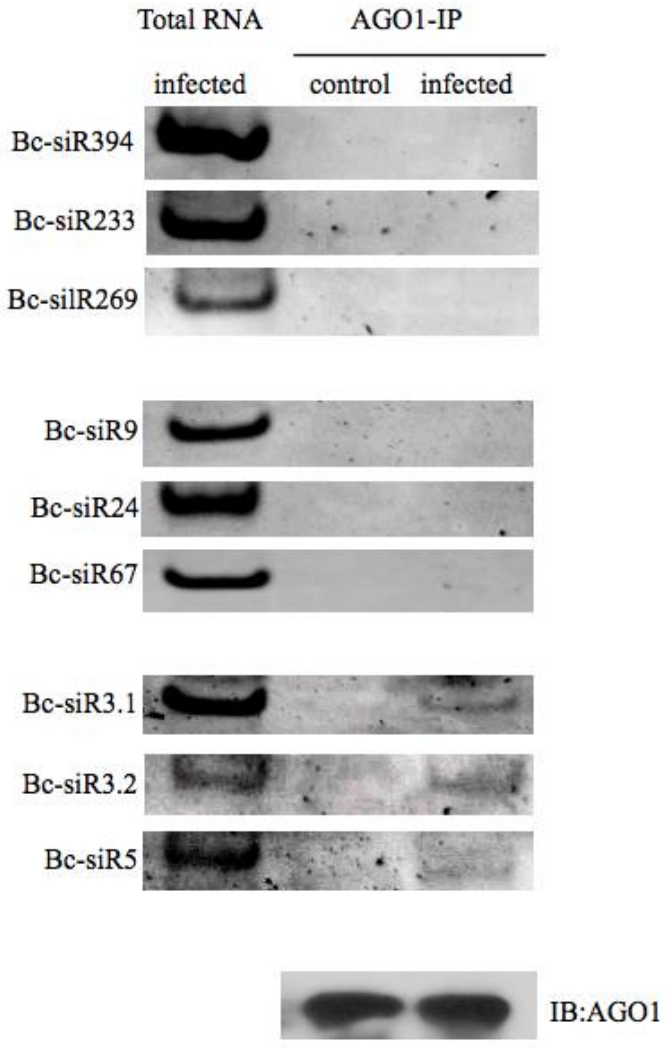

Figure 1.10 *Arabidopsis ago1-27* is more resistant to *B. cinerea* infection than wild-type

A. *ago1-27* displayed reduced disease phenotype upon *B. cinerea* infection. B. Induction of *BIK1* in response to *B. cinerea* infection was increased in *ago1-27*.

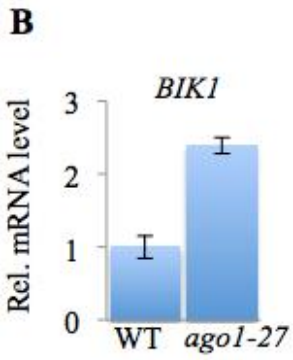

Figure 1.11 The phylogenetic tree of DCL proteins in pathogenic fungi

Schizosaccharomyces pombe and *Neurospora crassa* were used as references. An oomycete pathogen *Phytophthora infestans* was also included.

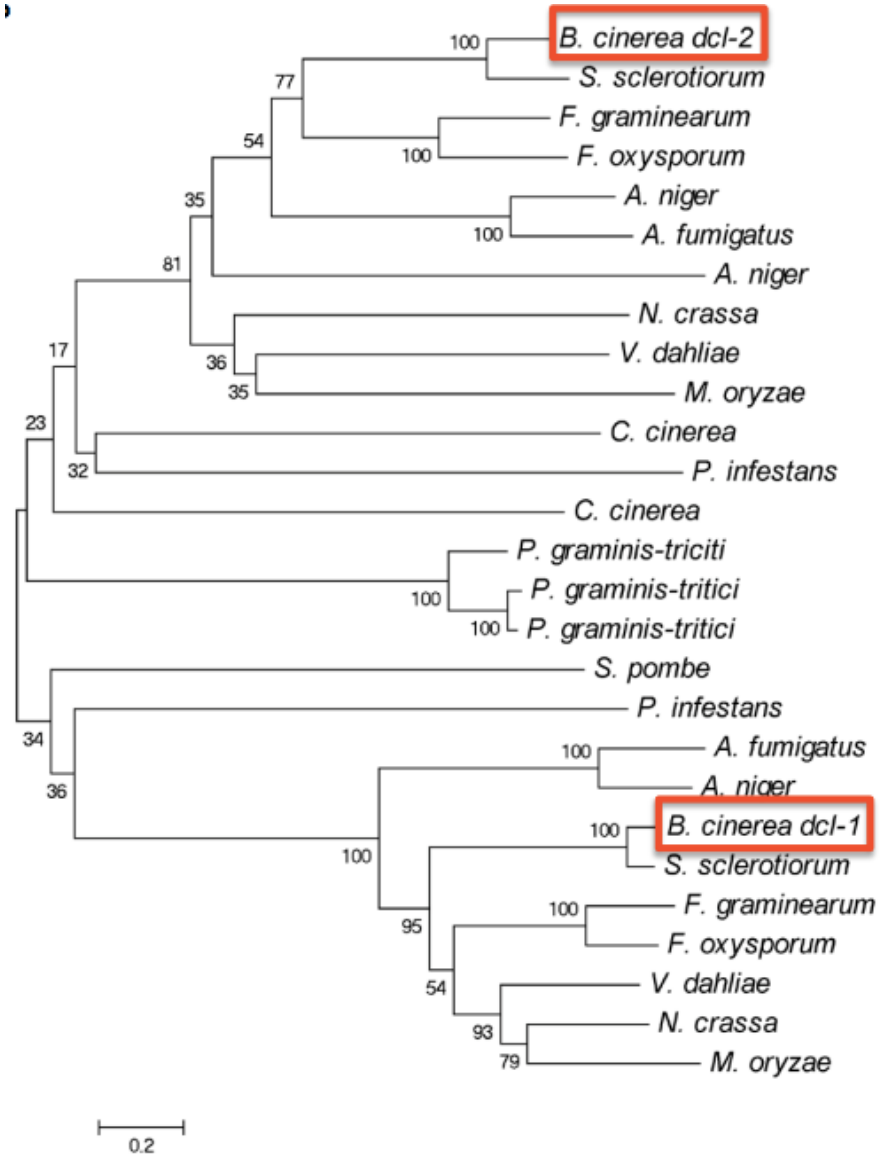

Figure 1.12 Generation of *B. cinerea dcl1, dcl2* single mutants and the *dcl1 dcl2* double mutant by homologous recombination

A. Schematic diagram of *Bc-DCL1* and *Bc-DCL2* knockout strategy by homologous recombination. Black arrows indicate primers used for genotyping. B. The *dcl1, dcl2*, and *dcl1 dcl2* knockout strains were confirmed by RT-PCR. C. *B. cinerea dcl1, dcl2*, and *dcl1 dcl2* mutant strains showed gradual growth retardation and delayed development of conidiospores: upper panel shows radial growth after 3 days, bottom panel shows conidiation at 21 days. D. Two Bc-sRNAs, Bc-microRNA-like RNA2 (Bc-milR2) and Bc-siR1498, were identified as Dicer-independent and were expressed in *dcl1 dcl2*.

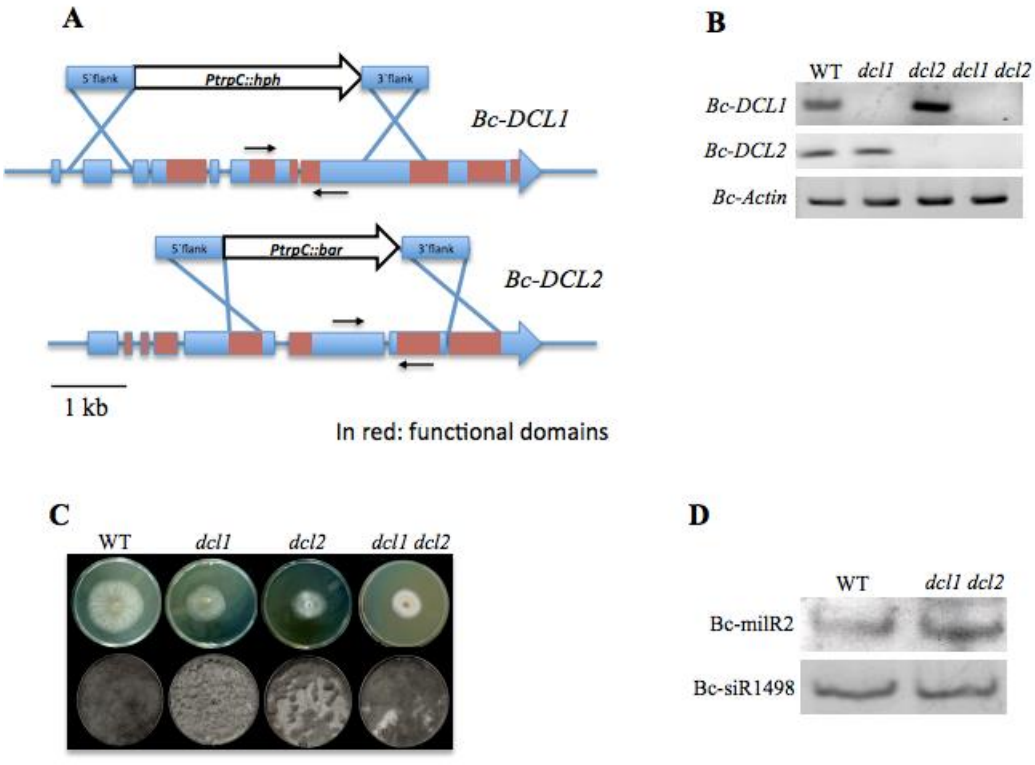

Figure 1.13 *B. cinerea dcl1 dcl2* double mutant is compromised in virulence

(**A**) *B. cinerea dcl1 dcl2* double mutant, but not *dcl1*or *dcl2* single mutants, was impaired in generating 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. (**E**) *B. cinerea dcl1 dcl2* mutant was compromised in suppression of *MPK2*, *MPK1*, and *PRXIIF* in *Arabidopsis* and *MAPKKK4* in *S. lycopersicum*. Similar results were seen in two biological repeats. (**F**) *Arabidopsis* Bc-siR3.1ox and Bc-siR3.2ox lines were more susceptible to *B. cinerea dcl1 dcl2* strain than was Col-0 wild type. (**G**) 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; $P < 0.01$).

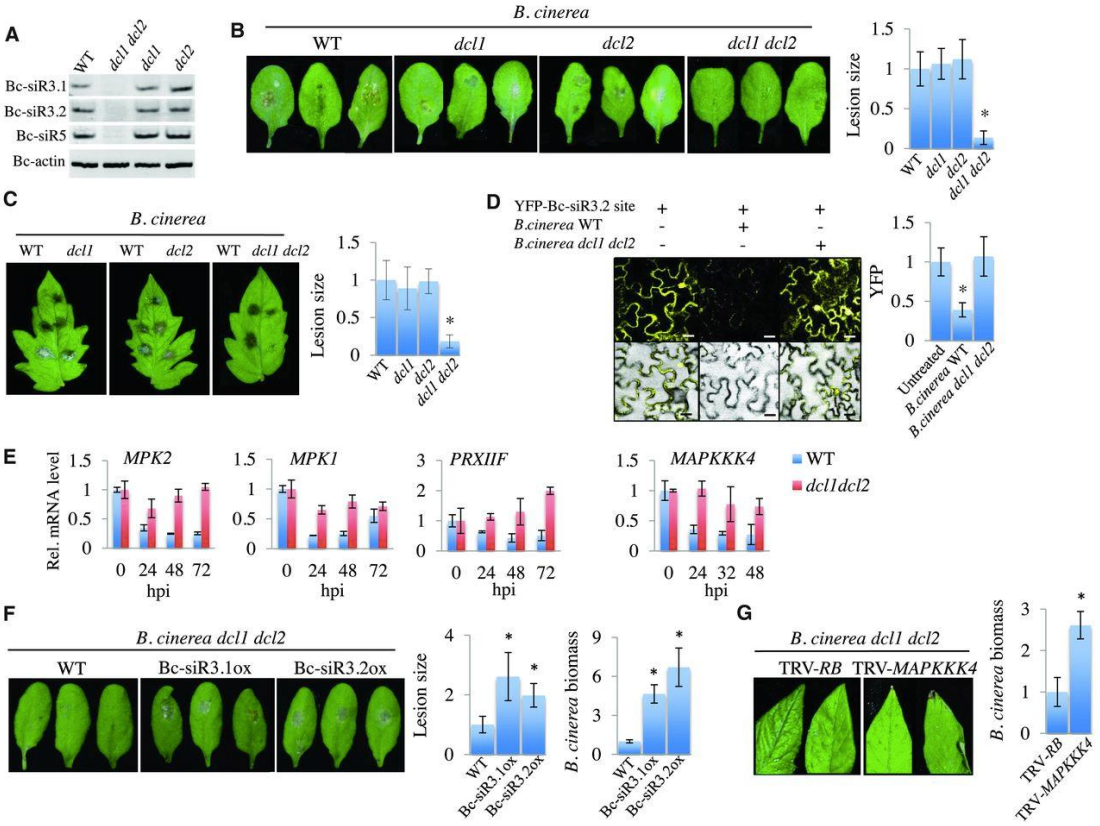
A
WT
dcl1 dcl2
dcl1
dcl2
Bc-siR3.1
Bc-siR3.2
Bc-siR5
Bc-actin
B
B. cinerea
WT
dcl1
dcl2
dcl1 dcl2
Lesion size
C
B. cinerea
D
YFP-Bc-siR3.2 site
B.cinerea WT
B.cinerea dcl1 dcl2
YFP
Untreated
E
MPK2
MPK1
PRXIIF
MAPKKK4
Rel. mRNA level
hpi
WT
dcl1dcl2
F
B. cinerea dcl1 dcl2
Bc-siR3.1ox
Bc-siR3.2ox
B. cinerea biomass
G
TRV-RB
TRV-MAPKKK4

Figure 1.14 *B. cinerea dcl1 dcl2* mutant is less virulent that wild-type strain on both Arabidopsis and tomato

The biomass of the *B. cinerea dcl1 dcl2* mutant strain was strongly reduced as compared with the wild-type strain during infection of both *Arabidopsis* (A) and *S. lycopersicum* (B), as quantified by qPCR at 72 hpi and 48 hpi, respectively.

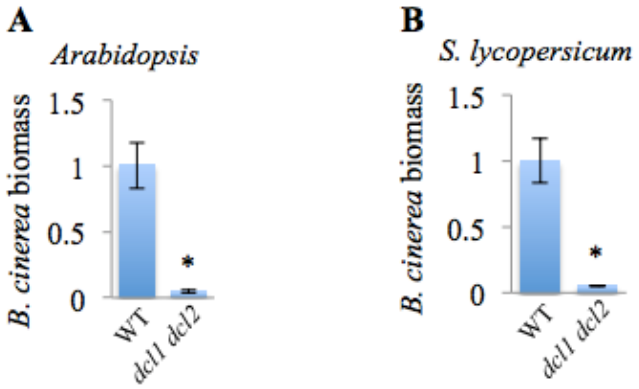

Table 1.1 Statistical analysis of the sRNA libraries from cultured *B. cinerea*, *B. cinerea*-infected *Arabidopsis*, and *B. cinerea*-infected *S. lycopersicum*

Library	Total reads	Total reads *B. cinerea*	% *B. cinerea* reads
Arabidopsis, 0 hpi (*B. cinerea*)	71,793,267	68,811	0.14
Arabidopsis, 24 hpi (*B. cinerea*)	101,220,872	609,204	0.65
Arabidopsis, 48 hpi (*B. cinerea*)	59,594,013	296,764	0.53
Arabidopsis, 72 hpi (*B. cinerea*)	41,478,258	338,325	0.82
S. lycopersicum leaf, 0 hpi (*B. cinerea*)	2,630,614	623	0.02
S. lycopersicum leaf, 24 hpi (*B. cinerea*)	1,586,314	6,315	0.28
S. lycopersicum leaf, 72 hpi (*B. cinerea*)	1,580,667	5,918	0.37
S. lycopersicum fruit, 0 hpi (*B. cinerea*)	6,334,100	1,381	0.02
S. lycopersicum fruit, 24 hpi (*B. cinerea*)	6,021,895	14,908	0.25
S. lycopersicum fruit, 72 hpi (*B. cinerea*)	3,617,356	458,590	12.68
B. cinerea, *in vitro* culture, conidiospores	787,441	787,441	100.0
B. cinerea, *in vitro* culture, mycelia	1,716,701	1,716,701	100.0
B. cinerea, *in vitro* culture, total biomass	18,086,243	18,086,243	100.0

Table 1.2 The predicted host targets of Bc-siR3.1, Bc-siR3.2, and Bc-siR5

Normalized read counts are given in reads per million *B. cinerea* sRNAs. Reads were summed from individual sRNA libraries for each category: cultured *B. cinerea*, *B. cinerea*-infected *Arabidopsis, B. cinerea*-infected *S. lycopersicum*. Target gene alignment was scored as described in Materials and Methods.

ID	Normalized read counts *Arabidopsis*	*S.lycopersicum*	*B.cinerea*	Alignment	Score	Predicted target gene
Bc-siR3.2	202	997	33	Bc-siR3.2 3'UGGAUGUUCUAGGUGUUACAU target 5'AUCAAGAAGAUCCACAAUGUG	3.0	**At MPK2**
				Bc-siR3.2 3'UGGAUGUUCUAGGUGUUACAU target 5'AUCAAGAAGAUUCACAAUGUU	4.5	**At MPK1**
				Bc-siR3.2 3'UGGAUGUUCUAGGUGUUACAU target 5'AUCUAGAAGAUCCAAAAUGUA	3.5	Sl F-box
				Bc-siR3.2 3'GUGGAUGUUCUAGGUGUUACA target 5'CAUUUAAAAGAUCCACCAUGU	4.5	**Sl MPKKK4**
Bc-siR3.1	812	1231	50	Bc-siR3.1 3'CGGGUGGAUGUUCUAGGUGUU target 5'AUCCACAUACAAGAUCCACAA	2.5	At Aminotransferase-like
				Bc-siR3.1 3'CGGGUGGAUGUUCUAGGUGUU target 5'GUCCCCUUACAACAUCCACAA	4.0	At Microspore-specific
				Bc-siR3.1 3'CGGGUGGAUGUUCUAGGUGUU target 5'GCCUAGCUACAAGAGCCACAU	4.5	**At PRXIIF**
				Bc-siR3.1 3'CGGGUGGAUGUUCUAGGUGUU target 5'AUCCACUUUCAAGAUCCACAG	4.25	Sl Autophagy ATG2-like
				Bc-siR3.1 3'CGGGUGGAUGUUCUAGGUGUU target 5'ACCCACCUGCAACAUCCACGA	4.5	Sl Vacuolar protein-sorting
Bc-siR5	1,710	1,380	303	Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'UAGGAAACUUUCCGAGUCAAA	4.0	At Unknown
				Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'GAGUUUGCAUUCCGGGUCGAA	4.0	At Clathrin, heavy-chain
				Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'GGGUAUACAUUCCGGGUCAGG	4.25	**At Cell wall-associated kinase**
				Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'GAAUUUACAAUCCGAGUCAAA	4.5	At MADS transcription factor
				Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'CAGUAUAGAUUCCGUGUCAAA	4.0	Sl TOM34
				Bc-siR5 3'UUCAUAUGUAAGGCUCAGUUU target 5'AGGUAGACAUUCUGAGGCAAA	4.5	Sl Pentatricopeptide

Table 1.3 The list of Bc-sRNAs that have predicted targets in both *Arabidopsis* and *S. lycopersicum*. (excel file)

Normalized read counts are given in reads per million *B. cinerea* sRNAs. Reads were summed from individual sRNA libraries for each category: cultured *B. cinerea*, *B. cinerea*-infected *Arabidopsis*, *B. cinerea*-infected *S. lycopersicum*. Target gene alignment was scored as described in Materials and Methods.

See http://www.sciencemag.org/content/342/6154/118/suppl/DC1 Table S3

Table 1.4 List of primers

primer	sequence	purpose
amiR319a oligo A	CTGCAAGGCGATTAAGTTGGGTAAC	Artificial microRNA cloning
amiR319a oligo B	GCGGATAACAATTTCACACA GGAAACAG	Artificial microRNA cloning
Bc-siR3.2 I miR-s	gaTACATTGTGGATCTTGTAGGTtctctcttttgtattcc	Artificial microRNA cloning
Bc-siR3.2 II miR-a	gaA CCTACAAGATCCACAATGTAtcaaagagaatcaatga	Artificial microRNA cloning
Bc-siR3.2 III miR*s	gaA ACTACAAGATGCACAATGTAtcacaggtcgtgatatg	Artificial microRNA cloning
Bc-siR3.2 IV miR*a	gaTACATTGTGCATCTTGTAGT Ttctacatatatattcct	Artificial microRNA cloning
Bc-siR3.1 I miR-s	gaTTGTGGATCTTGTAGGTGGGCtctctcttttgtattcc	Artificial microRNA cloning
Bc-siR3.1 II miR-a	gaGCCCACCTACAAGATCCACAAtcaaagagaatcaatga	Artificial microRNA cloning
Bc-siR3.1 III miR*s	gaGCACACCTACAAGTTCCACATtcacaggtcgtgatatg	Artificial microRNA cloning
Bc-siR3.1 IV miR*a	gaATGTGGAACTTGTAGGTGTGCtctacatatatattcct	Artificial microRNA cloning
Bc-siR5 I miR-s	gaTTTGACTCGGAATGTATACTTtctctcttttgtattcc	Artificial microRNA cloning
Bc-siR5 II miR-a	gaAAGTATACATTCCGAGTCAAAtcaaagagaatcaatga	Artificial microRNA cloning
Bc-siR5 III miR*s	gaAAATATACATTCCCAGTCAATtcacaggtcgtgatatg	Artificial microRNA cloning
Bc-siR5 IV miR*a	gaATTGACTGGGAATGTATATTTtctacatatatattcct	Artificial microRNA cloning
At-MPK2 F	CACCATGGCGACTCCTGTTGATCCAC	Gene cloning
At-MPK2 R	AAACTCAGAGACCTCATTGTTGTTTATGGTAGC	Gene cloning
At-MPK2 mutated version F	ATAAAGAAAATACATAACGTTTTTGAGAATAGGATTGATGCGTTGAGGACTC	Gene cloning
At-MPK2 mutated version R	AACGTTATGTATTTTCTTTATCGCCACTCTCTCATTACTCTCTCTGTTAAC	Gene cloning
At-MPK1 F	CACCATGGCGACTTTGGTTGATCCTCCT A	Gene cloning
At-MPK1 R	GAGCTCAGTGTTTAAGGTTGAAGCTTGTG	Gene cloning
At-MPK1 mutated version F	ATAAAGAAAATACATAACGTTTATGAGAATAGGATCGATGCGTTGAGGAC	Gene cloning
At-MPK1 mutated version R	AACGTTATGTATTTTCTTTATAGCAACTTTCTCGTTGGTGTCACTG	Gene cloning
At-WAK F	CACCATGAAAATCTTGATCTTGATTCTATCCTTTGTG	Gene cloning
At-WAK R	TCGCTGTCTTCTCTGAAAGCCTA	Gene cloning
At-WAK mutated version F	TGGAATTCACTCGGGCTCTGGTCCACCCATGTGTTGTGGG	Gene cloning
At-WAK mutated version R	CCAGAGCCCGAGTGAATTCCAGGTTGTCTGTATCCGACCATGT	Gene cloning
YFP-MPK2 target site F	CACCATGGTGAGCAAGGGCGAGGA	YFP-MPK2 site sensor cloning
YFP-MPK2 target site R	TTACACATTGTGGATCTTCTTGATCTTGTACAGCTCGTCCATGCCGA	YFP-MPK2 site sensor cloning
YFP-MPK2 target site mutated R	TTAAACGTTATGTATTTTCTTTATCTTGTACAGCTCGTCCATGCCGA	YFP-MPK2 site sensor cloning
Bc-DCL1 KO 3' flank F	ATCGTATTAATTAACTAGTTCGCCACAACCTGGCA	Gene knock out vector
Bc-DCL1 KO 3' flank R	ATCGTACTCGAGGGGCGATCGAAAAGCTTGCCA	Gene knock out vector
Bc-DCL1 KO 5' flank F	ATCGTGCCGCGGGGTACCCGGGAGACCCTGCCTCGTCCT	Gene knock out vector
Bc-DCL1 KO 5' flank R	ATCGTAAAGCTTTCTAGACTTCCAACTACCGTGGGTGCA	Gene knock out vector
Bc-DCL2 KO 3' flank F	ATCGTAAAGCTTTCTAGAGGCACGAAGGATATTTGTCGGC	Gene knock out vector
Bc-DCL2 KO 3' flank R	ATCGTACTCGAGGGCAGTCCGGAAGCTTTTCAGAG	Gene knock out vector
Bc-DCL2 KO 5' flank F	ATCGTATTAATTAATCTCCTGCCTTGGGGTCAAGA	Gene knock out vector
Bc-DCL2 KO 5' flank R	ATCGTACCGCGGGGTACCCGGAGGAGTAGACGAAGTCTGGC	Gene knock out vector
Bc-Pgdp-R check	ACTGGCTCTTAATGAGCTGGCG	Gene knock out vector check
Bc-TrpcC-F check	AACACCCAATACGCCGGCCGA	Gene knock out vector check
BAR-KpnI-F	GTCGCAGGTACCtcgacagaagatgatattgaaggagc	Gene knock out vector
BAR-BamHI-R	GTCTGAGGATCCGACGGATCAGATCTCGGTGACG	Gene knock out vector
Bc-DCL1 F check	ACGGCGCCCAGGAAGGGAGCTAGA	Genotyping PCR
Bc-DCL1 R check	AAGTCTGAGCTCACCTCCATCA	Genotyping PCR
Bc-DCL2 F check	GACCGACTATCCAGGACCATCCTCA	Genotyping PCR
Bc-DCL2 R check	TGCTCTGCCACCAATTGTACCGAT	Genotyping PCR
To-MPKKK4-attB1 F(VIGS)	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGACGACCCAAAGTCAAGAGAA	VIGS cloning
To-MPKKK4-attB2 R(VIGS)	GGGGACCACTTTGTACAAGAAAGCTGGGTTATGCAAGTCCAGACAGAATTTG	VIGS cloning
ToMPKKK4 RT F(VIGS)	TGTGTGGGCTCAGAAGAAGT	VIGS gene expression check
ToMPKKK4 RT R(VIGS)	ACAGGGCTCTCTATCCCAAC	VIGS gene expression check
Bc-siR3.1 northern blot probe	GCCCACCTACAAGATCCACAA	Northern blot
Bc-siR5 northern blot probe	AAGTATACATTCCGAGTCAAA	Northern blot
Bc-siR3.2 northern blot probe	ACCTACAAGATCCACAATGTA	Northern blot
At-MPK2 F (gene expression)	ACCGATAGGCCGAGGCGCGTA	Real-time PCR
At-MPK2 R (gene expression)	TTCAGATCCCGATGGAGAATG	Real-time PCR
At-MPK1 F (gene expression)	CACCTGGGATGTCTTTATCCAGAC	Real-time PCR
At-MPK1 R (gene expression)	CATCTCCTCTCTCAAATCCTCATCTAC	Real-time PCR
At-PRXIIF F (gene expression)	CGGGCCACGGTCTGAGAGATG	Real-time PCR
At-PRXIIF R (gene expression)	GATCTGTCCTAAGATGACTTC	Real-time PCR
At-WAK F (gene expression)	AGTGATGCGTTTTGTCGTGCGTGTG	Real-time PCR
At-WAK R (gene expression)	CTTGATGATGCACCGGTTGGTGATA	Real-time PCR
At-PDF1.2 F (gene expression)	CTTGTTCTCTTTGCTGCTTTCGAC	Real-time PCR
At-PDF1.2 R (gene expression)	TAGTTGCATGATCCATGTTTG	Real-time PCR
At-BIK1 F (gene expression)	CTCCTAATCGTGGACAATCGGCTAGA	Real-time PCR
At-BIK1 R (gene expression)	GTCCTGAAGTTGTTGTAAGGCACGGA	Real-time PCR
SALK-089827 F	AACCATCGTGCTCGGTGGCA	Genotyping PCR
SALK-089827 R	AGAGATGTTGCGGCACGGCA	Genotyping PCR
At-actin F (gene expression)	CAGTGGTCGTACAACCGGTATT	Real-time PCR
At-actin R (gene expression)	GTCTCTTACAATTTCCCGCTCT	Real-time PCR

Table 1.4 continues

primer	sequence	purpose
Sl-MPKKK4 F (gene expression)	CACTAGTCACGGTCTGAAGTCTGAC	Real-time PCR
Sl-MPKKK4 R (gene expression)	GGGTTCAGGTCAAACGATGGGCTCA	Real-time PCR
Sl-actin F (gene expression)	TCTCAGTGGTGGCTCCACCAT	Real-time PCR
Sl-actin R (gene expression)	TTAGAAGCACTTTCTGTGGAC	Real-time PCR
Bc-actin F (gene expression)	GAGAGCGGTGGTATCCACGTCAC	Real-time PCR
Bc-actin R (gene expression)	CACTTGCGGTGGACAATGGAAGGT	Real-time PCR
Bc-siR3.1 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCCAC	Rev. transcription
Bc-siR3.1 F	GCGGCGGTTGTGGATCTTGTA	PCR
Bc-siR3.2 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCTAC	Rev. transcription
Bc-siR3.2 F	GCGGCGGTACATTGTGGATCT	PCR
Bc-siR5 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAGTAT	Rev. transcription
Bc-siR5 F	CTCGCTTTTGACTCGGAATG	PCR
Bc-siR1498 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACCA	Rev. transcription
Bc-siR1498 F	GCGGCGGGGTGTTGTGGTTTA	PCR
Bc-milR2 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACAAC	Rev. transcription
Bc-milR2 F	GCGGCGGGTCCAGTGGTAGGA	PCR
Bc-siR394 F	CTCGTATGACTAGGCTTTT	Rev. transcription
Bc-siR394 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGATGTCAGAT	PCR
Bc-siR233 F	CTCGTAATCCCCTACAAAT	Rev. transcription
Bc-siR233 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAAAAAGAAG	PCR
Bc-siR269 F	CTCGCTAGGGGCCTATAAA	PCR
Bc-siR269 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGGA	Rev. transcription
Bc-siR9 F	CTCGTATTTTATGATGAGC	Rev. transcription
Bc-siR9 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGATCTAAAAA	PCR
Bc-siR24 F	CTCGTATGATTGGTCCTC	Rev. transcription
Bc-siR24 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAGTCAAACA	PCR
Bc-siR67 F	CTCGTATAAATCGATCGGA	Rev. transcription
Bc-siR67 RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAAAAAAATT	PCR
sRNA PCR universal R	GTATCCAGTGCAGGGTCCGAGGT	
Bc-ITS Forward	TCGAATCTTTGAACGCACATTGCGC	or Bc biomass quantificatio
Bc-ITS Reverse	TGGCAGAAGCACACCGAGAACCT G	or Bc biomass quantificatio

Chapter 3

Plant immunity under attack by a fungal pathogen small RNA effector

Abstract

Plants are under constant threat by pathogens. Almost all pathogens produce and secrete low molecular effector proteins that interfere with host proteins to undermine plant immunity. Recently, a novel class of pathogen effector molecules, small RNAs, has been discovered in an aggressive fungal pathogen. *Botrytis cinerea* is a necrotroph plant pathogen that secretes small RNA effectors into host cells that hijack a plant RNA interference pathway to silence important host immunity genes. We identified and functionally characterized further host immune-related genes that are targets of a novel type of a *Botrytis* sRNA effector, termed Bc-siR37. Bc-siR37 was detected in *Botrytis*-infected *Arabidopsis thaliana* (Arabidopsis) and *Solanum lycopersicum* tissues. Remarkably, the Bc-siR37 sequence exhibited a tremendous high number of 29 *in silico* predictable host target genes in Arabidopsis and *S. lycopersicum* using stringent prediction criteria. Most of the predicted host targets resembled genes that have putative regulatory or signaling functions in stress response and plant immunity. We confirmed transcriptional suppression of four Arabidopsis target genes upon *B. cinerea* infection, the immune-related transcription factor *AtWRKY7*, a putative pectin-lyase encoding gene conferring powdery mildew resistance (*PMR*) in its loss-of-function mutant, *Atpmr6,* a putative leucine-rich repeat receptor kinase gene, *AtFEI2*, and the autophagy-related gene *AtATG5*. We further verified *in vitro* as well as *in vivo* that *AtWRKY7*, *AtPMR6*, and *AtFEI2* were *bona fide*

targets of Bc-siR37. Moreover, pathogen assays revealed that transgenic Arabidopsis plants expressing Bc-siR37 as well as T-DNA insertion lines of *AtWRKY7*, *AtPMR6*, and *AtFEI2* exhibited enhanced disease susceptibility to *B. cinerea*, supporting the note that these Bc-siR37 target genes indeed participated in plant defense against *B. cinerea*. We finally propose that prediction of host target genes using pathogen sRNA sequence information can be a powerful tool to identify novel plant immunity genes.

Introduction

In plants, two physically associated modes of immune response carry out plant defense against microbial pathogens. First, pathogen-associated molecular patterns (PAMPs) are recognized by plant microbial pattern recognition receptors (PRRs) that signal PAMP-triggered immunity (PTI). PRRs are sub-divided into receptor-like kinases (RLKs) and receptor like proteins (RLPs), and in the model plant Arabidopsis most of the 600 RLK and RLP family members are involved in plant immunity [1-5]. For instance, the *Botrytis-induced kinase1* (*BIK1*) is a receptor-like cytoplasmic kinase that has been found to positively regulate plant basal defense against *B. cinerea* [6]. In addition, two other RLKs, *Brassinosteroid insensitive 1-associated receptor kinase 1* (*BAK1*) and *suppressor of BIR1-1* (*SOBIR-1*), are involved in plant defense against *B. cinerea* [7]. In a molecular arms race, successful pathogens secrete dozens of effector proteins to modulate host physiology and undermine PTI, which has been well described in bacterial and oomycete pathogens [8-10]. While bacterial pathogens evolved molecular secretion systems for effector delivery, such as the type-III, the type-IV secretion systems, delivery and entry of oomycete and fungal effectors into host cells stays rather enigmatic. In a second layer of

immune response, plant species evolve pathogen strain-specific resistance (*R*) genes. Most *R*-genes encode for receptor-like proteins of the nuclear-binding leucine-rich repeats (NB-LRRs) class. In a counter-defense mechanism, NB-LRRs recognize pathogen effectors by direct or indirect contact, and induce a robust immune response, called effector-triggered immunity (ETI). [9,11,12].

Yet, another important class of immune-responsive proteins are DNA-binding transcription factors. The largest family of transcription factors involved in plant immunity is WRKYs [13,14]. WRKYs, can act as both negative and positive regulators of gene expression. WRKYs and are involved in the antagonistic cross talk between salicylic-acid (SA)-dependent and jasmonic-acid (JA)-dependent plant defense pathways. [13-15]. For instance, WKRY33 is a negative regulator of SA-responsive genes and therefore promotes expression of JA-responsive defense genes that turns WRKY33 into a positive regulator for defense against necrotroph pathogens including *B. cinerea* [16,17]. Moreover, T-DNA insertion lines of *WRKY3*, *WRKY4*, and *WRKY70* showed enhanced disease susceptibility to *B. cinerea* [18,19], indicating a rather sophisticated regulatory network that includes several WRKYs in defense against *B. cinerea*.

Besides DNA-binding transcription regulators, small RNAs (sRNA), such as small-inferring RNAs (siRNAs) and microRNAs (miRNAs), that trigger RNA interference (RNAi) are also important in regulation and fine-tuning of plant immunity genes [20-22]. Several plant endogenous siRNAs and miRNAs contribute to the regulation of PTI, such as Arabidopsis miR393, miR160, and miR167 [23,24], or ETI, such as natural antisense

(nat)-siRNAATGB2 [25]and Arabidopsis long-siRNA-1[26], or both PTI and ETI, such as miR393* [27]. While the regulatory role of endogenous plant sRNAs in plant immunity has been broadly characterized, little is known about the role of sRNAs in microbial pathogenicity and host adaptation. Remarkably, many pathogens transmit sRNA effectors into the host cell, where they trigger host gene silencing that modulate host physiology and immune response. For instance, *B. cinerea* delivers sRNA effetors (Bc-sRNAs) that were shown to hijack the host AGO-RNAi machinery to silence important host immunity genes [28-30]. Bc-sRNA has been found to target important immune-related genes in two host plant species, Arabidopsis and *S. lycopersicum*. The Bc-siR3.2 targets the two related Arabidopsis *Mitogen-activated protein kinase 1* (*AtMPK1*) and *AtMPK2*, as well as the tomato *SlMPKKK4*. MPKs are important proteins in the signaling transduction of plant immune response. Most Bc-sRNAs that revealed predicted host plant genes are derived from long-terminal repeat (LTR) retrotransposon loci of the *B. cinerea* genome. LTR retrotransposons have been associated with fast evolving genome regions. This might contribute to fast turnover of Bc-siRNA effectors in the molecular arms race in host pathogen coevolution. Hence, mobile sRNAs are non-cell autonomous RNA signals that can even exchange between species of different kingdoms, such as plants and microbes, and induce cross-kingdom RNAi [20,31,32]. Conversely, expression of antisense RNA in plants that exclusively target pathogen mRNAs is nowadays applied to silence target genes in plant pathogens and pests, referred to as host-induced gene silencing (HIGS). HIGS is a powerful tool to study gene functions in non-transformable pathogens, such as most obligate biotrophs, and is a promising tool for crop protection measures.

Here, we characterized a novel class of Bc-sRNA effector, Bc-siR37, which was predicted to target 29 plant immunity genes in Arabidopsis and *S. lycopersicum* under stringent prediction criteria. We confirmed that three Arabidopsis target genes, *AtWRKY7*, *AtPMR6* and *AtFEI2*, were silenced by Bc-siR37 *in vitro* as well as *in vivo* that affected the plant defense response against *B. cinerea*. Bc-siR37 might be multi-potential in suppressing host immunity in a wide range of host plants. Finally, analyzing pathogen sRNAs for their potential to target host genes is an alternative route to identify novel host genes involved in immune response.

Results

The *B. cinerea* small RNA Bc-siR37 is predicted to target a large number of diverse host plant genes.

Next generation sequencing data obtained from previously studied sRNA libraries of *B. cinerea*-infected Arabidopsis leaves at 0, 24, 48, 72 hours post inoculation (hpi) and *B. cinerea*-infected tomato leaves and fruits (each 24 hpi and 72 hpi) led to the identification of three Bc-sRNAs that were proven to silence host immunity genes during the infection process [30]. Using our NGS data we identified a novel Bc-sRNA effector, termed Bc-siR37, which was detected in the sRNA libraries of both infected Arabidopsis and tomato tissues. The 21-nucleotide (nt) sequence of Bc-siR37 was uniquely mapped to the BC1G_10137 open read frame (ORF), which putatively encodes for an ABC-type plasma membrane ATPase (Figure 2.1). sRNAs derived from the entire BC1G_10137 ORF accumulated in moderate levels in the sRNA library obtained from *B. cinerea* mycelium

grown under axenic culture condition (non-infectious control) without showing any clear sRNA peak preference. However, especially Bc-siR37 was enriched in sRNA NGS data obtained from the conidiospore fraction of axenic-cultured *B. cinerea,* and, more intriguingly, in our *B. cinerea*-infected Arabidopsis tissue with a most predominant peak of Bc-siR37 at 24 hpi (Figure 2.1). Thus we interpreted this observation that Bc-siR37 might be rather functional during pathogenesis than a non-functional breakdown product of Bc1G_10137 transcripts.

Remarkably, we predicted a large number of potential host target genes in Arabidopsis and tomato by computational analysis of the 21-nt Bc-siR37 sequence. Applying stringent criteria, 15 target genes in Arabidopsis and 14 in *S. lycopersicum* were predicted (Table 2.1). Noteworthy, most predicted host target have putative regulatory or signaling functions related to plant immune response, such as LRR RLKs, ethylene-responsive factors, and WRKY transcription factors. Thus, we chose the Bc-siR37 and continued to evaluate the silencing capability on predicted host target candidates.

Arabidopsis Bc-siR37 target genes are silenced upon *B. cinerea* infection

We first confirmed the expression of Bc-siR37 during host infection. Arabidopsis-infected leave material was collected at 0, 24, 48, and 72 hpi, and adaptor ligation based PCR was applied to amplify Bc-siR37 (Figure 2.2A). A Bc-siR37 PCR band was clearly detected at 0, 24, 48, and 72 hpi, which verified our deep sequencing data. As a positive control for *B. cinerea* sRNA production *in planta*, we could detect Bc-siR3.2, a previously described sRNA effector of *B. cinerea* expressed during host plant infection (Figure 2.2A)

[30]. If Bc-siR37 would have a suppressive effect on host target genes during infection, we would speculate to find transcriptional suppression in host plants upon *B. cinerea* infection. Therefore, we performed real-time RT-PCR to quantify transcript levels of *AtATG5*, *AtWRKY7*, *AtFEI2* and *AtPMR6* upon *B. cinerea* infection. Transcript levels were measured at 0, 24, 48, and 72 hpi, according to detected Bc-siR37 expression in *B. cinerea*-infected Arabidopsis tissues (Figure 2.2A) and small RNA sequencing data (Figure 2.1). We observed that expression levels of all target genes evaluated here were clearly reduced at 24, 48, and 72 hpi compared to 0 hpi (Fig 2.2B). Further more, *AtPDF1.2*, a common *Botrytis*-induced marker gene showed a strong induction [33]. Therefore, we assumed that the reduced transcript levels of predicted Bc-siR37 targets was not due to cell damage caused by the pathogen infection, but were a result of a gene-silencing event. Nevertheless, large transcriptional re-programing during stress response is common in plants, which might also affect the transcription levels of the predicted target genes in Arabidopsis. Therefore, we worked towards finding further evidence for the Bc-siR37-silencing capability on the presumed host target genes.

Bc-siR37 efficiently silences Arabidopsis host immunity genes

A sequence stretch within the 5` UTR of *AtWRKY7* and *AtFEI2* transcripts revealed nearly-perfect complementary alignment with the 21-nt Bc-siR37 sequence, thus made them predicted target genes (Figure 2.3A). In order to find evidence for target site specific gene silencing, we conducted an *Agrobacterium tumefaciens*-mediated transient co-expression assay of Bc-siR37 with its host target genes using *Nicotiana benthamiana*

leaves. We cloned the Bc-siR37 sequence into a plant artificial microRNA vector (amiR-Bc-siR37), and *AtWRKY7* and *AtFEI2* cDNA sequences into the pEarlygate101 (pE101) expression vector that tagged hemagglutinin (HA) and YFP onto the C-terminal part of the gene of interest. To test target site sequence specificity of gene silencing, we cloned a sequence-mutated version of *AtWRKY7* (*AtWRKY7*m) and *AtFEI2* (*AtFEI2*m) target sites. Silencing efficiencies of Bc-siR37 on *AtWRKY7* and *AtFEI2* were analyzed 48 hours post *Agrobacterium*-infiltration by Western blot using an anti-HA antibody (Figure 2.3B). AtWRKY7 and AtFEI2 expressed well, when Bc-siR37 was not co-expressed. In contrast, when co-expressing Bc-siR37, a clear reduction of AtWRKY7 and AtPMR6 signals were visible, indicated a gene-silencing event. Moreover, when co-expressing the Arabidopsis miRNA AtmiR395, which had no sequence homology towards *AtWRKY7* or *AtPMR6*, no target gene suppression was observed. Finally, when co-expressing Bc-siR37 with AtWRKY7m and AtFEI2m, the silencing effect that was shown for the native gene version, was completely abolished (Figure 2.3A).

We verified the silencing efficiencies of Bc-siR37 for *YFP-AtWRKY7* in co-infiltrated tobacco leaves using quantitative image analysis of confocal microscopy pictures. Again, silencing was only observed, when Bc-siR37 was co-expressed with AtWRKY7, but not with AtWRKY7m (Figure 2.3B). We also infected YFP-AtWRKY7 or YFP-AtWRKY7m expressing tobacco leaves with *B. cinerea* and found that only AtWRKY7 was suppressed at site of fungal infections at 24 hpi, but not AtWRKY7m (Figure 2.3B). Based on these results, we assumed that Bc-siR37 efficiently silenced the Arabidopsis host genes *AtWRKY7* and *AtFEI2*.

Transgenic Bc-siR37-expression in Arabidopsis attenuates defense against *B. cinerea*

Upon finding evidence for gene silencing of host target genes by Bc-siR37 in tobacco leaves, we further on aimed to understand, if Bc-siR37 was capable to silence host target genes in the native plant species, Arabidopsis. Therefore, we transformed the amiR-Bc-siR37 expression vector into Arabidopsis Col-0 plants for stable expression. We obtained in total thirteen transformed lines that expressed of Bc-siR37 at different levels, as shown by Northern blot analysis (Figure 2.4A). None of the transformed lines exhibited any obvious morphological or developmental defects. We collected F2 generation progenies from highly Bc-siR37 expressing (Bc-siR37^{ox}) lines. In order to study the silencing effect of constitutively expressed Bc-siR37 on predicted Arabidopsis target genes, we measured transcriptional levels of *AtWRKY7*, *AtFEI2*, and *AtPMR6* in the Bc-siR37^{ox} line 3 in comparison to non-transformed wild type plants using real-time RT-PCR. In consistence to the results found in tobacco leaves (Figure 2.3), all tested Arabidopsis target genes were constitutively suppressed (Figure 2.4B). We thus assumed that Bc-siR37 was capable to suppress host target genes in Arabidopsis.

We next examined, if host gene silencing observed in the Bc-siR37^{ox} line 3 might alter the disease susceptibility towards *B. cinerea* infection. We drop-inoculated Bc-siR37^{ox} line 3 and wild type plants with *B. cinerea* spore suspension and observed enhanced disease susceptibility in the Bc-siR37^{ox} line 3 upon 4 hpi (Figure 2.4C). We assessed the disease severity by measuring the area of lesion formed by *B. cinerea*. These results pointed to a functional role of Bc-siR37-silenced host target genes in plant defense against this pathogen. This notion was supported by the fact that T-DNA insertion lines of *AtWRKY7*,

AtFEI2, and *AtPRM6* all showed enhanced disease susceptibility towards *B. cinerea* infection (Figure 2.5).

Discussion

Our previous discovery illustrated that Bc-siR3.1, Bc-siR3.2 and Bc-siR5 could act as effectors that hijacked host RNAi machinery to suppress host immunity genes. A list containing 73 Bc-sRNA effector candidates and their predicted host target genes in Arabidopsis and tomato was generated. Most of those potential Bc-sRNA effectors were originated from transposon region. In this chapter, we characterized a coding sequence derived Bc-siRNA effector Bc-siR37, which was not selected previously due to low read number, could also act as an effector to silence host target genes and contribute to *B. cinerea* pathogenicity. Bc-siR37 caught our attention because it had many more host target genes that were potentially involved in plant immunity than any other previously predicted Bc-sRNA effectors. One of the Arabidopsis target gene *AtATG5* has previously been demonstrated to regulate plant defense response against *B. cinerea* [34]. In addition, we also confirmed another three selected *Arabidopsis* genes *AtWRKY7*, *AtFEI2* and *AtPMR6* that took part in plant defense response against *B. cinerea.*

Unlike most predicted Bc-sRNA effectors, which are derived from retro-transposon regions, Bc-siR37 is derived from the gene coding sequence that encoding BcATPase BC1G_10137. Intriguingly, two *B. cinerea* ATPase genes, BcCCC2 and BcPMR1, have been proven to be pathogenicity genes [35,36]. The perspective was that BcATPase

BC1G_10137 might engage in *B. cinerea* pathogenicity not only through its own unknown regulation pathway, but also generating Bc-siR37 effector to suppress host immunity.

AtWRKY7 was found to negatively regulate SA-dependent *PR1* gene during plant defense response to bacterial pathogen *P. syringae* [33], which suggested that this gene might positively regulate JA defense pathway because of the antagonist relationship between SA and JA hormone signaling pathways. Consistently, our result indicated a positive defense role for AtWRKY7 in response to the necrotroph *B. cinerea*, which mostly trigger JA-dependent defense. However, whether AtWRKY7 regulate JA pathway directly or indirectly through inhibiting of SA signaling still need to be clarified. Another WRKY, AtWRKY33, a positive regulator of plant immunity against *B. cinerea*, has been suggested to indirectly regulate JA pathway [16,17]. However, whether this gene also positively contributes to plant immunity against other necrotrophs is still unknown. Interestingly, *AtWRKY57* was also a target of Bc-siR37. This gene was firstly identified as a mediator of plant drought tolerance [37]. Later, it was found that AtWRKY57 took part in JA pathway via interacting with JASMONATE ZIM-DOMAIN4/8 (JAZ4/8) [38], which suggests that *AtWRKY57* is involved in plant defense against *B. cinerea*. Therefore, Bc-siR37 could target in Arabidopsis different members of a gene family. The same phenomenon also occurs in tomato target genes, the ethylene responsive transcription factors (Table 2). Similarly, we had found earlier that Bc-siR3.2 targeted the homologous *AtMPK1* and *AtMPK2* genes [30] Taken together, Bc-sRNA effector might regulate a single pathway through silencing homologous genes to eliminate functional redundancy, or silencing different target genes for the purpose of strengthen suppression efficiency.

AtFEI2 is a transmembrane domain containing LRR-RLP, which has been implicated in cellulose synthesis but not in plant defense [39,40] Here, we identified its positive role in plant immunity against *B. cinerea*. However, since the cell wall integrity is very important for plant basal defense to *B. cinerea*, it remains a mystery whether AtFEI2 contributes to plant immunity by maintaining plant cell wall integrity or by performing the typical recognition function of a PRR. The amino acid sequence of AtFEI2 has an 82% homology to AtFEI1, and only *fei1fei2* double mutant, but not the single mutants, affected cellulose synthesis indicating redundant functions [41]. Considering that in our studies the *Atfei2* single mutant was more susceptible to *B. cinerea,* it is likely involved in a different pathway than the cellulose synthesis.

AtPMR6 is a pectin lyase that maintains pectin degradation activity. It was surprising that *Atpmr6* mutant was resistant to powdery mildew isolates but susceptible to other pathogens such as *P. syringae* pv. tomato and the oomycete *Hyaloperonospora arabidopsidis* [42]. Our data also confirmed the susceptibility of this mutant against the necrotrophic fungal pathogen *B. cinerea*. The reason why *Atpmr6* has contradictory impacts on different pathogens is unclear. Probably the defects in the cell wall are the reasons why this mutant reacts disparately to distinct pathogens.

All the predicted Bc-sRNA effector targets that we selected for functional characterization so far were involved in plant immunity against *B. cinerea*, including AtMPK1, AtMPK2, AtWAK, AtPRXIIF, AtATG5, AtWRKY7, AtFEI2, AtPMR6 and SlMPKKK4 [30]. Thus, we proposed a novel method to search for plant immunity genes through predicting the targets of pathogen sRNA effectors. This idea supposes that the

predicted host target genes of pathogen sRNA effectors were mostly relevant to plant immune system, providing a short cut for the investigation of plant genes in host pathogen interactions. As shown in Tables 2.1, Bc-siR37 has several host target genes with unknown functions, which could be neglected easily during reverse genetic studies. Our new proposed method could facilitate to solve this problem by providing sRNA effector host target list, including those with unknown functions, for verifying their roles in plant immunity. This method could be applied to the other pathogens that also secreted sRNAs as effectors.

Materials and Methods

Plasmid construction

amiR-Bc-siR37 construct was designed and generated according to the website WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi).

The sequence of Bc-siR37 was pasted in the MircoRNA sequence under oligo, and RS 300 (MIR319A *Arabidopsis thaliana*) was selected for vector backbone and run the program, which produced 4 oligos, (Bc-siR37 I miR-s, Bc-siR37 II miR-a, Bc-siR37 III miR*s, Bc-siR37 IV miR*a), plus the oligo A and oligoB present on the MIR319A vector, it has 6 primers for total (See Table S1 for sequence). The plasmid RS300 was used as template, Phusion DNA polymerase (Thermo Scientific) was used to amplify three fragments with primer pairs Oligo A and Bc-siR37 I miR (a), Bc-siR37 II miR-a and Bc-siR37 III miR*s (b), Bc-siR37 IV miR*a and Oligo B (c). The a, b, c fragments were used as templates, and together with primers Oligo A and Oligo B to amplify fragment d, which

exchanged Bc-siR37 into miR319a backbone. Fragment d was cloned into pENTR/D-TOPO (life science), and next to destination vector pEG100 by LR reactions.

AtWRKY7, AtWRKY7m, AtFEI2, and AtFEI2m over expression vectors: *Arabidopsis* cDNAs were used as templates, primers AtWRKY7-FOR and AtWRKY7-REV, AtWRKY7m-FOR and AtWRKY7-REV, AtFEI2-FOR and AtFEI2-REV, AtFEI2m-FOR and AtFEI2-REV, were added to amplify the cDNA sequences of AtWRKY7, AtWRKY7m, AtFEI2, and AtFEI2m, respectively. Phusion DNA polymerase (Thermo Scientific) was applied. AtWRKY7, AtWRKY7m, AtFEI2, and AtFEI2m cDNA fragments were cloned into pENTR/D-TOPO (life science), and next to destination vector pEG101 by LR reactions.

Plant materials

Arabidopsis thaliana (ecotype Columbia), Arabidopsis mutants and *N. benthamiana* were growing on 22°C under 12h light every day. All mutants were in Col-0 background. *Atwrky7* mutant line was provided by Dr. Zhixiang Chen's lab, *Atpmr6* and *Atfei2* T-DNA insertion lines are CS6580, and SALK_083958, respectively.

Bc-siR37ox lines: AmiR-Bc-siR37 construct was transformed into *A. tumefaciens* GV3101 by electric shock. The positive transformants of AmiR-Bc-siR37 in *A. tumefaciens* was selected and cultured at liquid LB with 50μg/ml kanamycin, 50μg/ml rifampicin and 100μg/ml gentamycin, at 28°C shaker overnight. *A. tumefaciens* was centrifuged at 4000RPM 15min at room temperature to collect bacterial pellets, which were resuspended in transformation buffer (50g Sucrose and 4.31g 1/2MS salt per L), 100 μl

0.1M acetosyringone and 25 µl/125ml silwet L-77 solution were added. The bacterial suspension was used for Arabidopsis dipping flowers for 1min. The transformed plants were wrapped with plastic membrane to keep moisture, and uncovered in the next day. Seeds were collected from transformed plants and transformants were screened by basta spraying.

Adaptor ligation-based PCR method to amplify Bc-siR37

Total RNAs from pure *B. cinerea* mycelium, and *B. cinerea* infected Arabidopsis under 0, 24, 48, 72 hours were extracted by TRIzol reagent (Life Techonologies). RNAs were running on 14% RNA denature polyacrylamide PAGE gel and stained by ethidium bromide. sRNAs at 18-30nt in length were cut from the gel, and purified with 0.4M NaCl at 4°C overnight. The purified sRNAs were ligated to 3' RNA adaptor with truncated T4 RNA ligase 2 (NEB, Ipswich, MA). Run the ligation products on the gel and cut RNAs from 30-50nt. The ligation products were purified and ligated with 5' RNA adaptor by T4 RNA ligase 1 (NEB, Ipswich, MA), and formed the final ligation products. Run the final ligation product on RNA gel and cut RNAs from 60-80nt. Purified the RNA ligation products and conducted reverse transcription (Life Technologies, Carlsbad, CA) to convert them into cDNAs at (50°C 60min, 70°C 15min). Amplify the cDNAs with adaptor specific primers SBS5' and SBS3' by touch down PCR to achieve DNAs (94°C 3min; 5cycles of 94°C 30s, 54°C 30s, 72°C 30s; 17cycles of 94°C 30s, 60°C 30s, 72°C 30s; and 72°C 10min). The DNA size was about 116nt, which were purified and used as template for Bc-siR37 PCR. Bc-siR37 lib forward primer and 3' adaptor reverse primer SBS3' were used

to amplify Bc-siR37. The size of the amplified product was about 80-bp determined by a PAGE. Primer sequences are in Table 2.2.

Check mRNA levels of target genes by Real-time PCR

Total RNAs were extracted by TRIzol reagent (Life Technologies, Carlsbad, CA), and treated by DNaseI enzyme (Roche) for 30 min at 37°C. The treated RNAs were purified by an extra RNA extraction by TRIzol. 1.5ug of clean RNAs were applied for reverse transcription by using SuperScriptIII kit (Life Technologies, Carlsbad, CA). The cDNAs were diluted 10 times for real-time PCR and reaction performed using Bio-Rad IQ5 machine (Bio-rad) with iQ SYBR Green Supermix (Bio-Rad) and RT primers. The thermos cycles were: 95°C 2min; and 45 cycles of 95°C 30s, 55°C 30s, 72°C 30s, florescent was measured at the step of 55°C 30s. All reactions had three replicates.

Measure the protein expression level by western blot

Proteins were extracted by grinding leaf tissues in 1.5 tubes with pestles followed by adding PB buffer [50mM Tris-HCl (pH6.8), 10%glycerol, 2%SDS, 5mM DTT, 1 tablet of protein inhibitor cocktail, BPB]. The protein extracts were boiled for 5min before running on the protein gel. The proteins on the gel were transferred to PVDF (Millipore) membrane for western blot. Membrane was blocked with 5% milk for 1 hour, HA-HRP (Santa Cruz Biotechnology) antibody in 5% milk (1:2000) were applied and incubated for 2 hours, and washed 4-times in TBST washing buffer for 5min each. Membranes were stained by ECL (GE Healthcare) for 1 min, exposed to an X-ray film in the dark room, and films were developed using an X-ray developing machine.

Transient expression/co-expression assay

A. tumefaciens carrying amiR-Bc-siR37, amiR395, AtWRKY7, AtWRKY7m, AtFEI2 and AtFEI2m were cultured separately in LB on 28°C shaker overnight. Cells were centrifuged to collect bacterial pellets and resuspended with infiltration buffer (10mM MgCl2, 10mM MES, and 0.2 mM acetosyringone). The OD600 was adjusted to 1.0 and cells were kept on room temperature for 4 hours before infiltration. Cells were infiltrated into leaves of 4 weeks old *N. benthamiana*. Two days after infiltration, leaf samples were collected for protein extraction or confocal microscopy. *B. cinerea* infestation of *N. benthamiana* will be described below.

Measure sRNA levels by northern blot

RNA samples were boiled for 5 min and run on 14% RNA denatured polyacrylamide gel at 150v for 5h, then RNAs were transferred into Amersham Hybond-N+ (GE health) membrane. RNAs were chemically cross-linked with membrane by EDC 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich). Bc-siR37 probe was labeled with gamma ATP-p32 radioisotope (PerkinElmer) and used to probe the RNAs on the membrane overnight. Membranes were washed in 0.1%SDS in 1×SSC buffer for 20min three times. Membranes were exposed to phosphorscreens, and scanned by typhoon PhosphorImager.

B. cinerea infection assay

10 days old *B. cinerea* spores were collected. The concentration was calculated by hemacytometer and spores were diluted to 2×10^5 in B5 inoculation medium (10 mM Sucrose, 10 mM KH2PO4, Tween-20 0.025%.) for infection 15 µl spore solution were dropped on the center of 4-week-old Arabidopsis leaves. After 4 days, photos were taken to record the results and imageJ was used to measure the lesion size. For the infection of *N. benthamiana* that transiently expressed AtWRKY7 and AtWRKY7m, two-days after Ago-infiltration, *B. cinerea* spores were inoculated at the bottom of the infiltrated leaves. After 1 day post-inoculation, the infected areas of the leaves were cut and YFP signal was examined using confocal microscope Leica SP2 (Leica).

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Figures and Tables

Figure 2.1 Bc-siR37 had a dominant peak in *Arabidopsis* infected libraries, but not in pure *B. cinerea* library

Total RNAs were extracted from pure *B. cinerea*, as well as *B. cinerea* infected *Arabidopsis* for 0, 24, 48, and 72 hours. These RNAs sample were used for sRNA libraries construction, and followed by next generation deep sequencing. This figure showed the read distribution of sRNAs among the ATPase gene BC1G_10137 (Top panel) in all five sRNA libraries. The Bc-siR37 reads in all the libraries were marked.

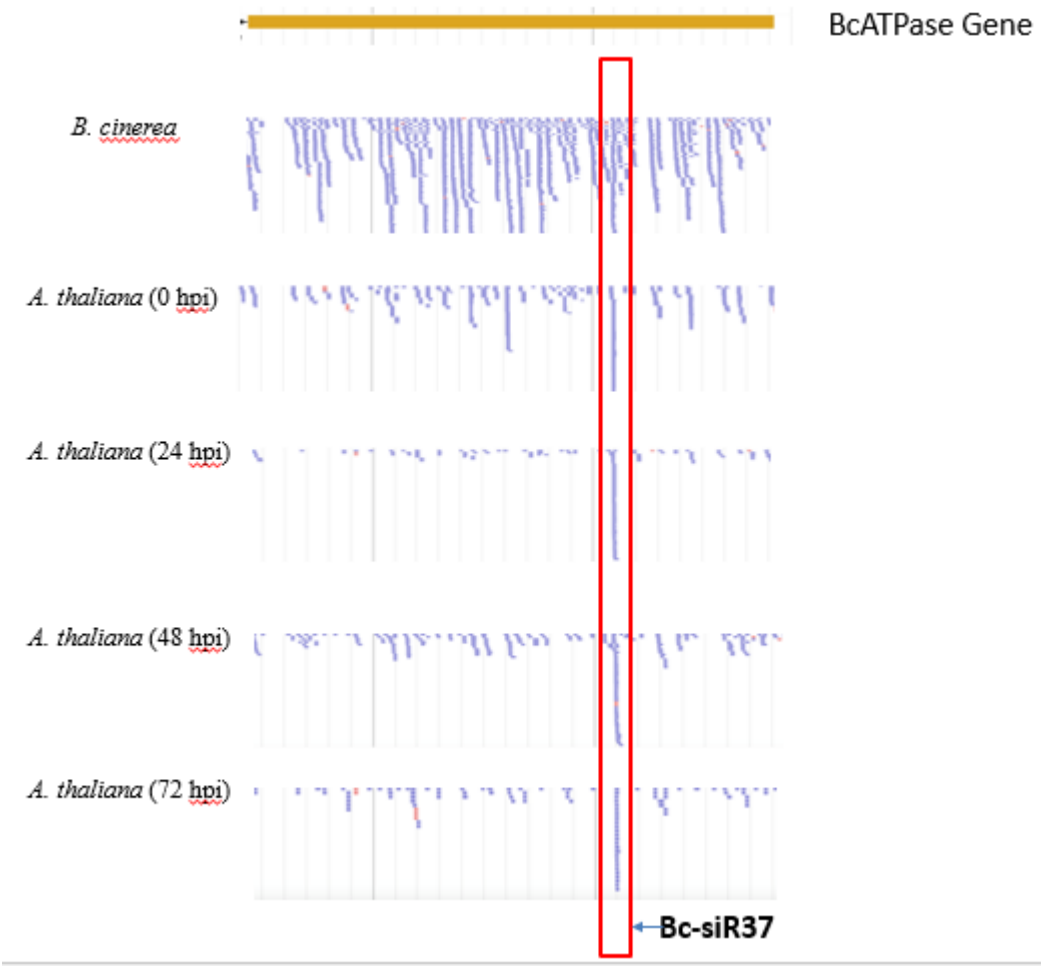

Figure 2.2 Bc-siR37 was induced whereas its host target genes were suppressed during different time courses *Arabidopsis* infestation

A) Total RNAs were extracted from *B. cinerea* as well as it infected *Arabidopsis* in 0, 24, 48, 72 hours. The sRNAs were purified and ligated with both 5' and 3' RNA adaptors. The ligated RNAs were converted to cDNAs by reverse transcription and Bc-siR37 was detected by PCR amplification. Both *Arabidopsis* and *B. cinerea* actin were amplified with the cDNAs that transcribed from the same total RNAs as Bc-siR37. B) qRT-PCR was used to analyze the relative mRNA levels of representative *Arabidopsis* targets *AtWRKY7*, *AtPMR6*, *AtFEI2* and AtATG5 during *B. cinerea* infection at 0, 24, 48, and 72 hours. The mRNA level of the marker gene *AtPDF1.2* was used as a control.

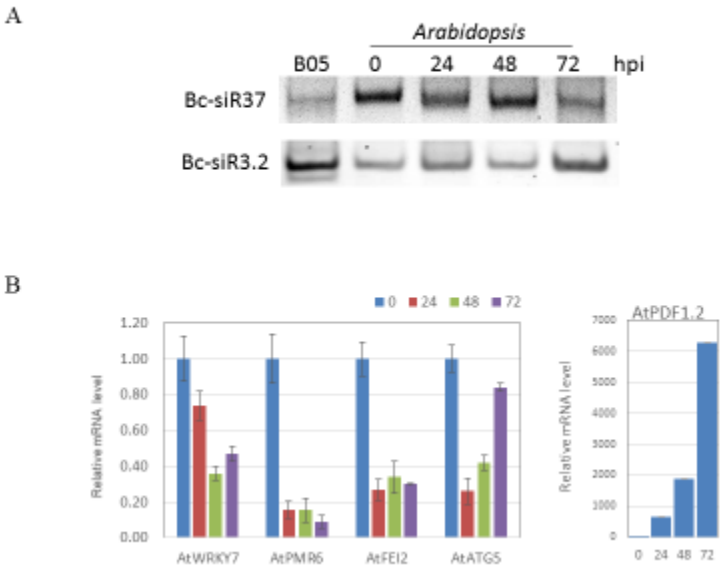

Figure 2.3 AtWRKY7 and AtFEI2 were specifically down regulated by Bc-siR37

A) Co-expression of Bc-siR37 and its targets AtWRKY7 (top panel) or AtFEI2 (Bottom panel) in *N. benthamiana* indicated the suppression of target genes by Bc-siR37. The expression levels of the targets were measured by western blot. Co-expression of Bc-siR37 with mutated targets, and co-expression of At-miR395 with targets were used as controls. B) Transiently expressed YFP tagged AtWRKY7 and AtWRKY7m in *N. benthamiana* leaves, which were treated with or without *B. cinerea* 2 days after agroinfiltration. YFP signals of the infected or non-infected leaves were measured under microscope after one-day *B. cinerea* infection (top panel), scale bar indicates the YFP intensity.

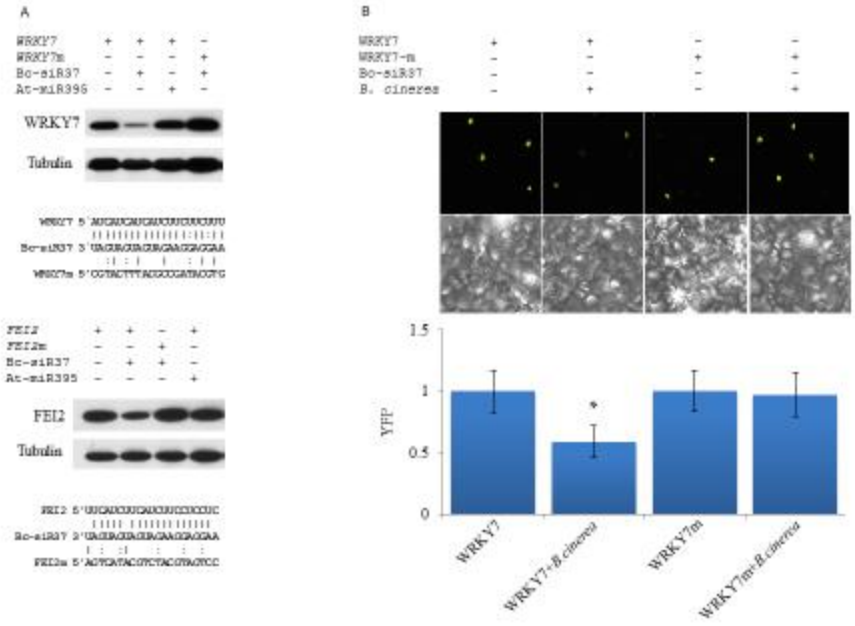

Figure 2.4 *Arabidopsis* Bc-siR37 transgenic lines showed lower mRNA levels of the target genes and more susceptible to *B. cinerea*

A) Total RNAs were extract from 13 Arabidopsis Bc-siR37 transgenic lines as well as wild type plants. Bc-siR37 expression levels in these lines were analyzed by Northern blot, and the highly expressed lines were used for further experiments. U6 was used to indicate the loading of each sample. B) The transgenic line Bc-siR37ox3 was selected for qRT-PCR to measure the mRNA levels of *AtWRKY7*, *AtPMR6* and *AtFEI2*. These targets were all suppressed in the Bc-siR37 transgenic lines. C) 4-week old Bc-siR37 transgenic plants from the first and second generation of line Bc-siR37ox3 were used for *B. cinerea* infection. The pictures were taken 4-days after infection, and the lesion sizes of individual leaves were calculated by imageJ (right panel). Error bars indicated the SD from 10 different leaves.

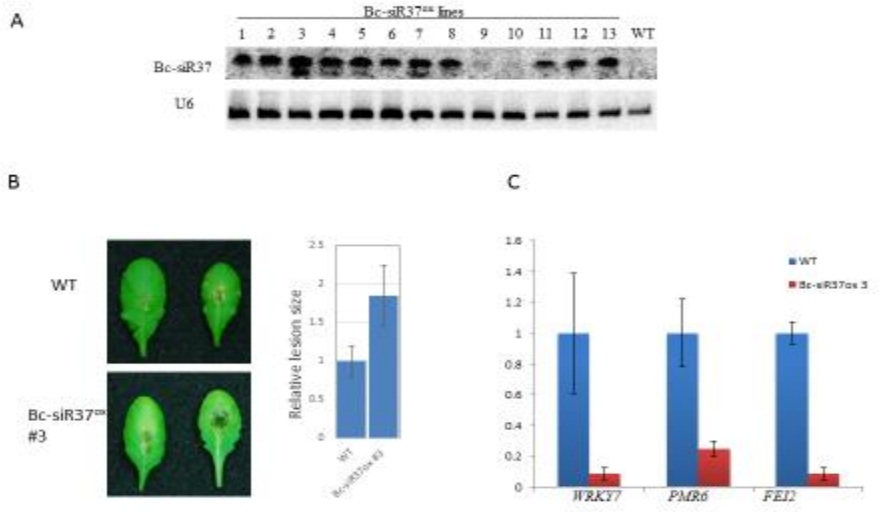

Figure 2.5 *atwrky7, atfei2 and atpmr6* mutant plants were more susceptible to *B. cinerea*

Four-week old Arabidopsis Col-0 wild type and *atwrky7, atfei2 and atpmr6* mutant plants were used for *B. cinerea* infection. The mutants were more susceptible to *B. cinerea*, when compared with wild type plants. The pictures were taken 4 days after infection, and the lesion sizes were calculated by imageJ indicated by the bar scale (right panel). Error bars represent the SD of 10 different infected leaves.

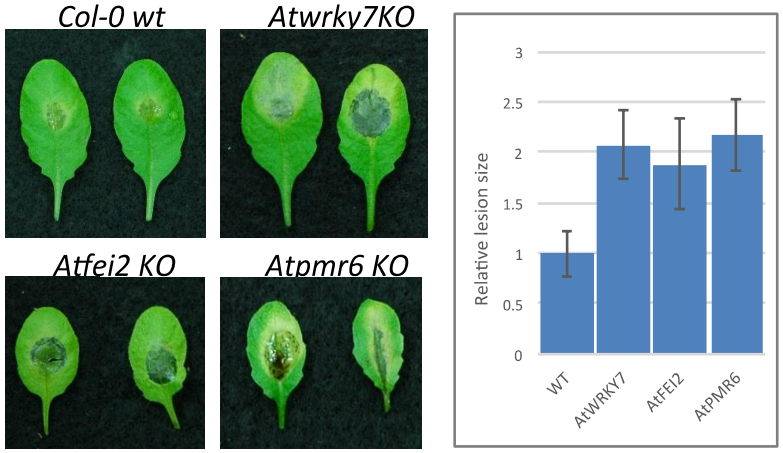

Table 2.1 Bc-siR37 host targets in both *Arabidopsis* and *S. lycopersicum*

Bc-siR37 *A. thaliana* target genes			Bc-siR37 *S. lycopersicum* target genes		
Target gene ID	Alignment score	Putative function of target gene	Target gene ID	Alignment score	Putative function of target gene
AT4G24240	2	**WRKY7**	Solyc07g026680	2	MYB transcription factor
AT1G69310	4	WRKY57	Solyc09g091950	3	Ethylene-responsive transcription factor 1
AT2G18350	3	HB24 homeo-box	Solyc05g050830	3	Ethylene-responsive transcription factor 4
AT2G35620	3	**LRR kinase, FEI2**	Solyc10g080540	3	Homeobox-leucine zipper protein 14
AT5G35980	3	YAK1-like kinase	Solyc04g079830	4	BEL1-like homeodomain protein 2
AT1G68330	2	Protein Kinase	Solyc06g005280	3	EXO70
AT3G54920	4	**Pectin lyase, PMR6**	Solyc01g108440	2	Calmodulin-binding protein
AT3G51830	4	ATG5	Solyc08g082470	3	Harpin-induced protein
AT2G44410	4	RING/T-box	Solyc08g048430	4	F-box family protein
AT1G64220	3	TOM7-2 subunit	Solyc10g006350	4	Microtubule-associated protein TORTIFOLIA1
AT2G36460	4	FBA6	Solyc06g084600	2.5	unknown
AT1G02813	2.25	DUF538	Solyc10g006940	3	unknown
AT3G18050	3	unknown	Solyc06g072070	3.25	unknown
AT3G46880	2	unknown	Solyc11g068720	4	unknown
AT1G63057	3	unknown			

Table 2.2 List of primers

primer	sequence
Bc-siR37 seuence	AAGGAGGAAGAUGAUGAUGAU
Bc-siR37-PCR For	GTCCGACGATCAAGGAGGAAGA
Bc-siR3.2-PCR For	AGTCCGACGATCTACATTGTGGATC
3' Adaptor-PCR Rev	CAAGCAGAAGACGGCATACGAG
At-ACTIN2-For	AGTGGTCGTACAACCGGTATT
At-ACTIN2-Rev	GATGGCATGAGGAAGAGAGAA
At-PDF1.2 RT F	CTTGTTCTCTTTGCTGCTTTCGAC
At-PDF1.2 RT R	TAGTTGCATGATCCATGTTTG
ATG5-RT FOR	GTAAGGCAGGATGCTTTGGACCT
ATG5-RT REV	CACAACTCCTGCAGTCACTCCAG
WRKY7 RT FOR	CAAAATGGCTGATATACCATCAGATGA
WRKY7 RT REV	GCATGGTTGTGGTCTCCTTCG
PMR6 RT FOR	TCGTAGACCAGCTCACTCGAA
PMR6 RT REV	CATCTGGAAGACATTGCTGTCATC
Fei2 RT3 for	CGGTTACTTGGCTCCAGAGTATA
Fei2 RT3 rev	CTATTGATAGAAGTGCGTCGAGACT
B siRNA 37 I miR-s	gaAAGGAGGAAGATGATGATGATGATtctctcttttgtattcc
B siRNA 37 II miR-a	gaATCATCATCATCATCTTCCTCCTTtcaaagagaatcaatga
B siRNA 37 III miR*s	gaATAATATCATCATACCTCCTTtcacaggtcgtgatatg
B siRNA 37 IV miR*a	gaAAGGAGGTATGATGATATTATtctacatatatattcct
amiR319a oligo A	CTGCAAGGCGATTAAGTTGGGTAAC
amiR319a oligo B	GCGGATAACAATTTCACACA GGAAACAG
AtWRKY7 cloning FOR	CACCCACTCTCTTCATTTATTCTTCCCCTCATC
AtWRKY7M cloning FOR	CACCTCTTCCCCTTGACCGTA
AtWRKY7 cloning REV	AAGAGTTTTGTCATGATTCATCGTCGTCG
AtFEI2 cloning FOR	CACC CTGTCT TATTCTTCATCTTCATCTTCCTCCT
AtFEI2 cloning REV	ATCGGAGCTGGAGTCGTAGAAGTC
AtFEI2M cloning FOR	CACCCTGTCTAGCAGAGTCATACGTCTACGTAGTCCGCGGATCCATTTTGAGGAGTCTCT
Bc-siR37 probe	ATCATCATCATCTTCCTCCTT

Chapter 4

Host-induced gene silencing of *Botrytis cinerea dcl1 dcl2* enhanced the plant immunity against gray mold disease

Abstract

The gray mold disease on various fruits and vegetables is caused by a necrotrophic fungal pathogen *Botrytis cinerea*. This pathogen is so aggressive that can broadly infect more than 200 plant species. However no effector protein of this fungus has yet been reported. Recently, we reported that *B. cinerea* delivers a novel type of effector molecules, small RNA effectors, into the host cells to interrupt expression of host immunity genes, thus achieve better host plant colonization. The *B. cinerea dcl1 dcl2* double mutant was less virulent and failed to produce three long terminal repeat (LTR) retrotransposon-derived Bc-sRNA effectors, Bc-siR3.1, Bc-siR3.2 and Bc-siR5. Actually, 53 of 73 predicted Bc-sRNA effectors were LTR-retrotransposon origin. Here we showed that, like the model fungi *Neurospora crassa,* Bc-sRNAs has both *B. cinerea* Dicer like proteins (BcDCLs)-dependent and –independent biogenesis. In addition, we found that most of the transposon derived Bc-sRNAs were BcDCLs-dependent, which indicated that BcDCLs contributed to pathogenicity through regulating the production of most Bc-sRNA effectors. Therefore, we performed host-induced gene silencing (HIGS) against BcDCLs in both *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato), and demonstrated that these HIGS plants displayed a stronger defense response to *B. cinerea*. This chapter provides a RNA based method to manage the gray mold diseases.

Introduction

Botrytis cinerea is a very aggressive necrotrophic fungal pathogen that infects a broad range of plant species and causes the gray mold disease. The severity of damage it inflicts on crop plants results in an estimated $10 to $100 billion loss worldwide annually. Consequently, it was voted number two in the list of top 10 plant fungal pathogens with the most scientific and economical importance by an international community of fungal pathologists [1]. The attachment and infection of airborne *B. cinerea* conidia on the surface of plant leaves or fruits primarily causes tissue collapse and water-soaked lesions, followed by spreading of the lesions over the next several days [2,3]. It can cause disease in multiple growth stages of various plants. While most fungi are inhibited by low temperatures, *B. cinerea* is so aggressive that it can infect and cause serious disease symptoms on fruits or vegetables stored at cold temperatures. Currently, the most commonly used method to control *B. cinerea* is the use of fungicides [4,5]. The disadvantages of constant fungicide application, environmental pollution and fungicide insensitivity or resistance, drive the need to understand *B. cinerea* pathogenicity and to find an alternative method to controlling grey mold disease [6].

In the on-going arms race between host plants and pathogens, pathogens evolved effector molecules to inhibit the host immune system, and consequently, host plants evolved resistance (R) proteins that recognize these effectors and trigger immune responses, including the hypersensitive response (HR) [7-10]. Pathogen effector molecules that have been previously identified were all proteinaceous in nautre; however, our recent study has revealed that small RNAs (sRNAs) can also function as effectors to suppress host immune

pathways [11-13]. sRNAs are short, noncoding RNAs that mediate silencing of target gene expression, called RNA interference (RNAi). Mature sRNAs are loaded into argonaute (AGO) proteins to form a RNA-induced silencing complex (RISC), which is guided by the sRNA to complementary target genes to induce silencing post-transcriptionally either by mRNA degradation or translation inhibition or transcriptionally by DNA methylation and histone modification. Small-interfering RNAs (siRNAs) and microRNAs (miRNAs) are the two major sRNA classes that are conserved in most eukaryotes [14,15]. miRNAs are generated from pre-miRNAs that have a stem-loop hairpin structure, whereas siRNAs are processed from linear long double stranded RNAs [16-18].

Most plant and animal siRNAs and miRNAs are processed by RNaseIII domain-containing endoribonucleases, Dicer or Dicer-like (DCL) proteins [14,15]. In contrast, sRNA biogenesis in fungi is more complicated [19,20]. *B. cinerea,* like the model filamentous fungus *Neurospora crassa,* utilizes both DCL-dependent and DCL-independent sRNA biogenesis [11,21,22]. *B. cinerea* has two DCLs—BcDCL1 and BcDCL2, and several DCL-dependent sRNAs, including Bc-siR3.1, Bc-siR3.2 and Bc-siR5, have been identified [11]. The *B. cinerea dcl1 dcl2* double mutant has severely reduced pathogenicity to both Arabidopsis *thaliana* (Arabidopsis) and *Solanum lycopersicum* (tomato), implicating DCLs in host-pathogen interaction [11]. Although the *dcl* mutants of several other fungal pathogens, such *as Magnaporthe oryzae* and *Mucor circinelloides* [23-25], have defects in growth or sporulation, no mutants identified thus far have defects in pathogenicity. Another type III RNase enzyme, MRPL3, contributes to the processing of some DCL-independent microRNA-like RNAs (milRNAs) [21]. However,

the proteins that regulate the generation of DCL-independent siRNAs are still unknown. So far, two *B. cinerea* DCL-independent sRNAs, Bc-siR1498 and Bc-milR2, have been reported [11].

The role of host plant endogenous sRNAs in defense has been brought to light in the past several years. Many sRNAs have been identified as having specific roles in the reprogramming of immunity genes, as both negative and positive regulators, during pathogen attack [26-29]. Some sRNAs are upregulated, such as miR393 [30], miR160, miR167 [31], miR393* [32], nat-siRNAATGB2 [33], and AtlsiRNA-1 [34], while others are downregulated, such as miR398a and miR773 [31]. Artificially overexpressing siRNAs in host plants to target pathogen virulence genes, a tool called host-induced gene silencing (HIGS), has been proven to successfully enhance plant immunity against the pathogen [35-39]. This is an example of cross kingdom RNAi, in which the sRNAs move between organisms from different kingdoms [40-42]. HIGS has been widely used in various plants to defend against oomycete and fungal pathogens and insect pests. However, it is still unknown how the host siRNAs regulate target genes of these pathogens. One of the key steps in using HIGS is selecting a pathogen virulence-related gene for silencing. Generally, pathogen effectors are the best choice, because they are directly involved in host-pathogen interactions. *B. cinerea* only has Bc-sRNA effectors [11], as no protein effectors has been reported thus far. However, BcDCLs have also been demonstrated to contribute to *B. cinerea* pathogenicity since they are involved in producing Bc-sRNA effectors [11]. Therefore, HIGS targeting BcDCLs is likely to boost host defense responses to *B. cinerea*.

In this chapter, I present the recent advances in our study of *B. cinerea* sRNAs and their role in host plant interactions. First, I present our results from our global profiling of Bc-sRNAs in the wild type and *dcl1 dcl2* double mutant. We discovered that most transposon-derived Bc-sRNAs are BcDCLs-dependent. This is consistent with our previous data showing that most of the Bc-sRNA effectors originated from transposon regions. Then, we further confirm that BcDCLs contribute to *B. cinerea* pathogenicity since they are involved in the biogenesis of most of the Bc-sRNA effectors. Lastly, we performed HIGS on both Arabidopsis and tomato against BcDCLs and show that this approach successfully increases plant resistance against *B. cinerea*.

Results

BcDCLs regulate the production of most Bc-sRNAs originating from retrotransposon regions, which are hotspots for Bc-sRNA effectors.

Our previous data has indicated that BcDCLs regulate *B. cinerea* pathogenicity by processing Bc-sRNA effectors, such as Bc-siR3.1, Bc-siR3.2, and Bc-siR5, which previously have been characterized. However, two other Bc-sRNAs, Bc-siR1498 and Bc-miR2 appeared to be BcDCLs independent. In order to understand the relationship between BcDCLs, Bc-sRNA effectors, and *B. cinerea* pathogenicity, we performed global profiling of the sRNAs in both wild type *B. cinerea* and the *dcl1 dcl2* double mutant using next generation deep sequencing. Bc-sRNAs that were 20-35 nucleotide (nt) in length were selected for further analysis. The *dcl1 dcl2* double mutant had a reduced number of Bc-sRNAs reads in the 20- to 27-nt size range. However, the number of reads of Bc-sRNAs

longer than 27-nt was significantly higher than in the wild type. The longer reads possibly came from the accumulation of incompletely cleaved sRNAs by BcDCLs (Figure 3.1A). Overall, the *dcl1 dcl2* double mutant had a reduction of 38% Bc-sRNAs reads when compared with wild type *B. cinerea* (Table 3.1). This indicates that Bc-sRNAs are partially BcDCL-dependent similar to *N. crassa* [21].

Next, we independently compared the Bc-sRNA read numbers among five functionally distinct genomic regions: retrotransposon, ORF (Open Reading Frame), intergenic, tRNA, and rRNA. There were 2–4 times more reads in the ORF, intergenic, and tRNA regions in the *dcl1 dcl2* double mutant than in the wild-type, whereas there were approximately half the number of reads in the rRNA regions (Table 3.1, Figure 3.2). However, the most significant difference was in the retrotransposon regions: the number of reads in the *dcl1 dcl2* double mutant was 25-fold lower than in the wild-type (Table 3.1, Figure 3.1B) indicating that retrotransposon-derived Bc-sRNAs are BcDCLs-dependent. Particularly, Bc-siR3.1 and Bc-siR3.2 reads were not present in the *dcl1 dcl2* double mutant, while wild type levels were 337.24 and 289.73 Reads Per Million (RPM), respectively. In addition, Bc-siR5 had only 0.1 RPM in *dcl1 dcl2* double mutant yet 153.61 RMP in the wild-type (Figure 3.1C). This result was consistent with previous data showing that Bc-siR3.1, Bc-siR3.2, and Bc-siR5 were clearly detected in *B. cinerea* wild type, *dcl1*, and *dcl2* single mutants but not in the *dcl1 dcl2* double mutant [11].

The distribution of all the Bc-sRNAs reads in the wild-type revealed a peak from 24–26 nt, while the *dcl1 dcl2* double mutant had a more balanced distribution among all the sizes and slightly peaked from 25–29 nt and at 33 nt (Figure 3.1A). However, Bc-

sRNAs produced in retrotransposon regions shifted the peak to 21–22 nt (Figure 3.1B). Moreover, the majority of such transposon-derived Bc-sRNAs have a 5' terminal U (Figure 3.1D), a feature that targets the sRNA for loading into AGO1 protein in Arabidopsis [43,44]. Consistent with this, 63 of 73 Bc-sRNA effectors that have been identified also have this feature. This explains the phenomenon that the Arabidopsis *ago1-27* mutant is more resistant to *B. cinerea*. In conclusion, we further confirmed that BcDCLs regulate *B. cinerea* pathogenicity by producing most of the retrotransposon-derived Bc-sRNA effectors.

Host induced gene silencing of BcDCLs (HIGS-BcDCLs) in Arabidopsis increased plant defense responses to *B. cinerea*.

Our previous study has shown that the *B. cinerea dcl1 dcl2* double mutant was less virulent than the wild-type on both Arabidopsis and tomato. Our Bc-sRNA profiling results show that BcDCLs are responsible for the generation of Bc-sRNA effectors and are thus participate in host-pathogen interactions. Therefore, we reasoned that we could use HIGS in Arabidopsis to silence both BcDCLs in order to control grey mold disease. To this end, we expressed RNAi constructs in Arabidopsis Col-0 wild-type plants that target both *BcDCL1* and *BcDCL2* in order to inhibit the production of Bc-sRNA effectors and thus attenuate *B. cinerea* virulence. The RNAi fragments were designed to avoid the conserved functional domains of Arabidopsis DCL (AtDCL) proteins so as not to disturb their expression or function (Figure 3.3). Northern blot was performed to measure the expression levels of individual transformed plants (HIGS-BcDCLs) (Figure 3.4A), which are morphologically similar to wild-type plants. Three highly expressed lines together were

used for *B. cinerea* infection. Transgenic HIGS-BcDCLs plants showed less disease symptoms after *B. cinerea* drop inoculation, when compared with wild-type plants (Figure 3.4B), which indicated that the HIGS of BcDCLs indeed enhanced host resistance to *B. cinerea*.

Virus-induced gene silencing of BcDCLs (VIGS-BcDCLs) in *S. lycopersicum* also strengthened plant immunity against *B. cinerea*.

In order to confirm that the HIGS-BcDCLs-based method can be successfully applied in the field, we also used this method on the natural host of *B. cinerea,* tomato plants, from where *B. cinerea* was originally isolated. Because transient expression in tomato plants would take less time than stable transformation, we chose to perform VIGS-BcDCLs by co-agroinfiltration of the binary tobacco rattle virus (TRV) vectors (pTRV1 and pTRV2) in order to silence the BcDCLs. The selected RNAi fragments of BcDCLs were cloned into the pTRV2 vector. pTRV2 carrying a *Phytoene desaturase gene* (*PDS*) and a late-blight resistance gene (*RB*), that is not present in *S. lycopersicum*, were used as positive and negative controls, respectively.

After agroinfiltration of multiple leaves with the *PDS* positive control vector, the fifth, sixth, and seventh leaves exhibited the strongest bleached leaf phenotype, indicating the strongest silencing of the *PDS* gene. In Arabidopsis, disruption of *PDS3* caused an albino phenotype, which is attributed to the disruption of chloroplastic genes [45]. Therefore, we detached these three leaves from both VIGS-BcDCLs and VIGS-RB infiltrated plants and infected them with *B. cinerea* using spray inoculation. Three days

post inoculation (dpi), VIGS-RB leaves showed very severe collapse and water lesions, while the VIGS-BcDCLs leaves were less damaged (Figure 3.5A). Total RNA from these leaves was extracted and used for Northern blot to analyze the expression level of siRNAs against BcDCLs (siRBcDCLs). All the leaves showing less severe disease symptoms still expressed siRBcDCLs, although the levels varied (Figure 3.5B), which was consistent with the uneven silencing by VIGS in tomato. These results in tomato indicated again that expression of siRNAs against BcDCLs in the host could enhance resistance to *B. cinerea*.

Discussion

Our previous study has illustrated that the *dcl1 dcl2* double mutant decreased *B. cinerea* pathogenicity on both Arabidopsis and tomato [11]. We also showed that this double mutant fail to generate three Bc-sRNA effectors Bc-siR3.1, Bc-siR3.2, and Bc-siR5, which are delivered into host cells to hijack host RNAi machinery and suppress host immunity [11]. The function of the BcDCLs in processing sRNAs at the whole genome level was unknown. From these previous results, we speculated that they are involved in the biogenesis of at least some important Bc-sRNA effectors. Thus, we profiled sRNAs from *B. cinerea* wild-type and the *dcl1 dcl2* double mutant. We found that Bc-sRNAs were partially BcDCLs-dependent. Interestingly, most Bc-sRNAs generated from retrotransposons are BcDCLs dependent, and 52 of 73 Bc-sRNA effectors that we previously predicted were of retrotransposon origin. These results further demonstrated that BcDCLs contributed to the pathogenicity through production of most of the Bc-sRNA effectors. In addition, most transposon-derived Bc-sRNAs have a 5’ U and are 21–22 nt in

length, a feature for AtAGO1 loading in Arabidopsis [43,44], which explains why the Arabidopsis *ago1-27* mutant has enhanced resistance to *B. cinerea*.

Transposable elements have been very dynamic throughout evolution, thus they play an important role in the co-evolution of hosts and pathogens. Pathogen effectors and host plant R proteins were fundamental components during the host-pathogen arms race. Some pathogen effectors and host plant R proteins are located near transposons [46-48], allowing them to evolve more quickly and adapt to each other during their co-evolution. Therefore, the fact that most Bc-sRNA effectors are located in the retrotransposon regions is also an advanced mechanism for *B. cinerea* to infect a wide range of host plants. Transposon-derived Bc-sRNAs were mostly BcDCLs-dependent, thus also confirmed the role of BcDCLs in its pathogenicity. We believe that *B. cinerea* has more than the known 73 Bc-sRNAs, which were predicted using host target information from Arabidopsis and tomato. Since *B. cinerea* can infected over 200 plant species, if target genes of other host species were considered, more Bc-sRNA effectors could be identified. Therefore, we hypothesize that *B. cinerea* has a large sRNA effector pool which are selectively secreted during infection based on the host targets. Some effectors might be delivered into multiple host species against several conserved immune genes, while others might be only delivered into certain host species to target specific host genes. Future research may uncover whether this is true.

After functionally characterizing BcDCLs in *B. cinerea* pathogenicity, we validated a HIGS-BcDCLs-based method to control gray mold disease. HIGS has been largely applied to control oomycete and fungal pathogens and insect pests, by expressing dsRNAs

to silence pathogen or pest target genes and to inhibit disease symptoms. HIGS has been explored much more in parasitic biotrohpic pathogens than necrotrophic pathogens. Biotrophic fungi create a structure called haustorium in the host tissue during infection, which not only helps to absorb nutrients from the host but also allows delivery of effector proteins into the host cells to inhibit host defense responses. This might also be the means for biotrophs to uptake artificially expressed siRNAs from the host that silence pathogen target genes to inhibit the infection process. Necrotrophic pathogens directly release many cell-degrading enzymes to rupture the host cells and create a more straightforward and direct contact between the host and pathogen cells. However, the mechanism or efficiency of sRNA movement from host to necrotrophic pathogens is unknown. Furthermore, timing may be an issue for host cells as they must send the siRNAs into the pathogen before they themselves are killed. It is possible that this process occurs during the very early infection stage before the host cell is ruptured.

Spraying plants with fungicide is the most common traditional method used to treat gray mold disease. The advantage of this method is its high efficacy in limiting *B. cinerea* growth. However, this method is costly and is detrimental to the environment, especially in the long term. More importantly, it triggers fast evolution of fungicide-insensitive *B. cinerea* isolates. In this chapter, we discovered a novel method to control gray mold disease by stably expressing siRBcDCLs in the host plants.

Materials and Methods

Plasmid construction

For the pHELLSGATE8-BcDCLs plasmids, the RNAi fragments of BcDCL1 (252 bp from CDS 1965–2216) and BcDCL2 (238 bp from CDS 765–1002) were amplified from *B. cinerea* cDNAs using these primers: BcDCL1F: GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCGGAAGAACTTGAAGGTTTGC TACA and BcDCL1R: GTCCAGATCTGGTCAACACACCAAG; and BcDCL2F: CTTGGTGTGTTGACCAGATCTGGACGGATGCCATTTGCTGCACGC and BcDCL2R:GGGGACCACTTTGTACAAGAAAGCTGGGTACTCTTGAGTACTTTC GCCAGCTCAC, respectively. Overlapping PCR was performed with primers BcDCL1F and BcDCL2R to ligate the RNAi fragments of BcDCL1 and BcDCL2. The RNAi fragment amplification PCRs were done using Phusion DNA polymerase (Thermo Scientific, Carlsbad, CA). The ligated RNAi fragment was cloned into pDONR207 using Gateway® BP clonase technology (Life Technologies, Carlsbad, CA), and into the destination vector pHELLSGATE8 using Gateway® LR technology (Life Technologies, Carlsbad, CA).

For the pTRV2-BcDCLs plasmids, the pDONR207-BcDCLs vector was doing LR reaction with pTRV2 EV to get pTRV2-BcDCLs by LR clonase (Life Technologies, Carlsbad, CA).

Plant materials

Arabidopsis, *N. benthamiana*, and tomato plants were grown at 22°C under a 12-hr light cycle. All Arabidopsis were Columbia-0 ecotype and tomato were of cv. Moneymaker.

HIGS-BcDCLs transgenic plants: The HIGS-BcDCL pHELLSGATE8-BcDCL plasmid was transformed into *A. tumefaciens* strain GV3101. Transformed strains were grown on the LB plates and then cultured in liquid LB medium with 100μg/ml spectinomycin, 50μg/ml gentamycin, and 50μg/ml rifampicin, and shaken overnight at 28℃. The bacterial was centrifuged at 4000 RPM for 15 min, and the pellet was resuspended in transformation buffer (5% sucrose, 0.54g 1/2MS powder, 25μl silwet L-77). Flowering Arabidopsis wild-type plants with flowers were dipped in this bacterial solution. Seeds collected from the transformed plants were grown on 0.5X Murashige and Skoog (MS) plates with 100μg/ml kanamycin to select for positive transformed plants.

VIGS-BcDCLs in tomato plants: The pTRV2-BcDCL plasmid was transformed into *A. tumefaciens* strain GV3101. VIGS was performed by co-agroinfiltration of the binary TRV vector (TRV1 and TRV2 [49]) into two-week-old tomato cv. Moneymaker leaves. *A. tumefaciens* carrying TRV1 and TRV2-BcDCLs, TRV2-RB, or TRV2-PDS were cultured in liquid LB with 50μg/ml kanamycin, 50μg/ml gentamycin, and 50μg/ml rifampicin and shaken overnight at 28℃. The bacterial was centrifuged at 4000 RPM for 15 min, and the pellet was resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 0.2 mM acetosyringone). The OD600 values were adjusted to 1.0, and equal volumes of TRV1 and TRV2 strains were mixed before infiltration.

RNA extraction

The plant tissues were collected and frozen in liquid nitrogen and then ground into fine powder using mortars and pestle. RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacture's instructions.

Northern blot

RNA samples were run on 14% denaturing RNA gel and transferred onto Amersham Hybond-N+ (GE health) membrane. After chemically cross-linked the RNA with the membrane using EDC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma), the membrane was probed with gamma dCTP-p32 radioisotope labeled BcDCL RNAi fragment at 65℃ overnight. The membrane was then washed with 0.1% SDS in 1×SSC buffer for 3 times for 20 min at 65℃ before exposing to the phosphorscreens. Finally, the sRNA signal was detected using a Typhoon PhosphorImager.

***B. cinerea* infection assay**

The *B. cinerea* inoculum was prepared from 10-day-old *B. cinerea* grown on plates. The spores were first washed using sterile water and diluted in B5 medium (10 mM Sucrose, 10 mM KH2PO4, 0.025% Tween-20 to a final concentration of 1×10^4 spores/mL for tomato infection; 2×10^5 spores/mL for Arabidopsis. Arabidopsis plants were performed with drop infection by placing a 15 µl drop of spore solution onto the center of the plant leaves. For VIGS experiment in tomato plants, spray inoculation was used to evenly spread the *B. cinerea* spore solution onto the tomato leaves. The pictures were taken to record the disease symptoms 3–4 dpi.

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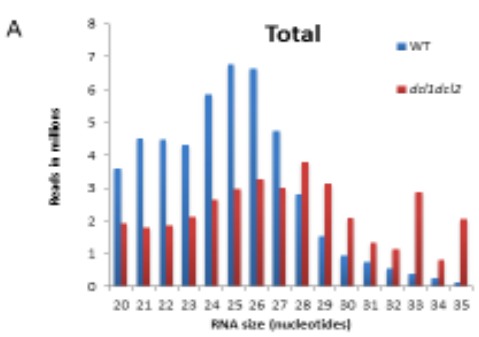
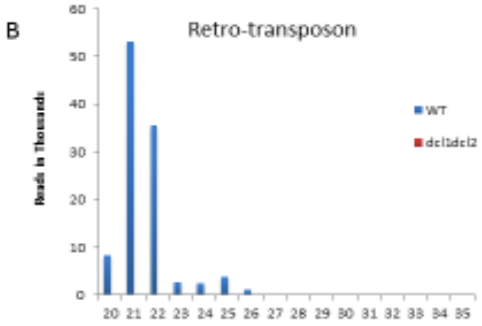
Figures and Tables

Figure 3.1 Retrotransposon-derived Bc-sRNAs are mostly BcDCL-dependent

Two libraries were constructed from wild type *B. cinerea* and the *dcl1 dcl2* double mutant and sequenced using Illumina deep sequencing. A) The read numbers of all Bc-sRNA reads from the two libraries according to sRNA size . B) The read numbers of retrotransposon-derived Bc-sRNA from the two libraries according to sRNA size. C) The normalized read numbers of Bc-siR3.1, Bc-siR3.2, and Bc-siR5 from the two libraries. D) The read numbers of retrotransposon-derived Bc-sRNAs according to 5' nucleotide (A,U, C, or G) and sRNA size. The X-axis in A, B, and D indicates RNA size in nucleotides.





C

	WT	*dcl1dcl2*
Bc-siR3.1	337.24	0
Bc-siR3.2	289.73	0
Bc-siR5	153.61	0.1

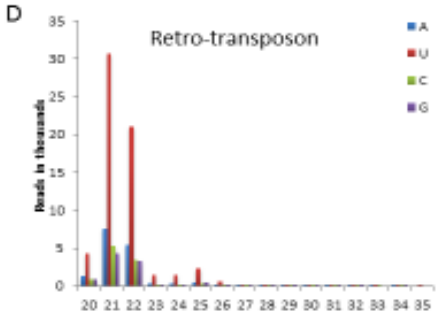


Figure 3.2 The read numbers of Bc-sRNAs among ORFs, intergenic, tRNA, and rRNA regions according to sRNA size

Two libraries were constructed from wild type *B. cinerea* and the *dcl1 dcl2* double mutant and sequenced using Illumina deep sequencing. The normalized read numbers of Bc-sRNAs from ORF (A), intergenic (B), rRNA (C), and tRNA (D) regions. The X-axis in A, B, and D indicates RNA size in nucleotides.

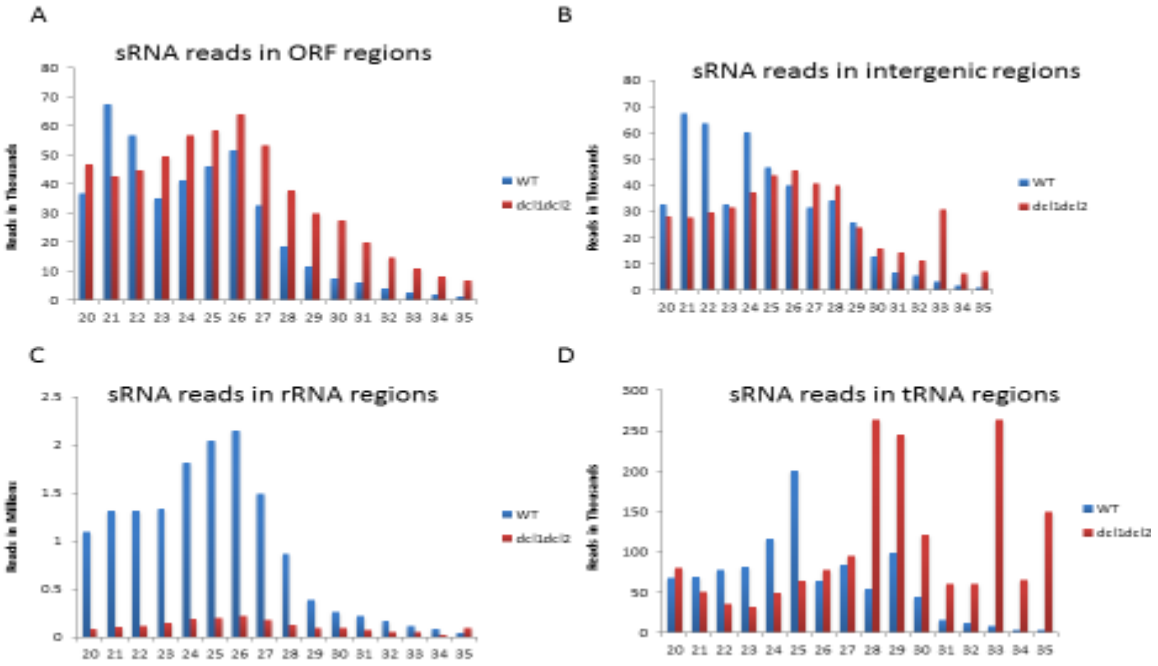


Figure 3.3 Alignment of amino acid sequences of the RNAi fragment region of *B. cinerea* DCLs and Arabidopsis DCLs

In order to avoid the target of the siRBcDCLs to any of the four Arabidopsis DCLs (AtDCLs), the amino acid sequence of BcDCL1 and BcDCL2 were aligned with AtDCL1–4. The RNAi fragments of both BcDCL1 and BcDCL2 were selected to avoid any conserved functional domains of the AtDCL proteins. The alignment of these six genes at the selected RNAi fragment loci were shown, and BcDCL1 and BcDCL2 RNAi fragments are highlighted.

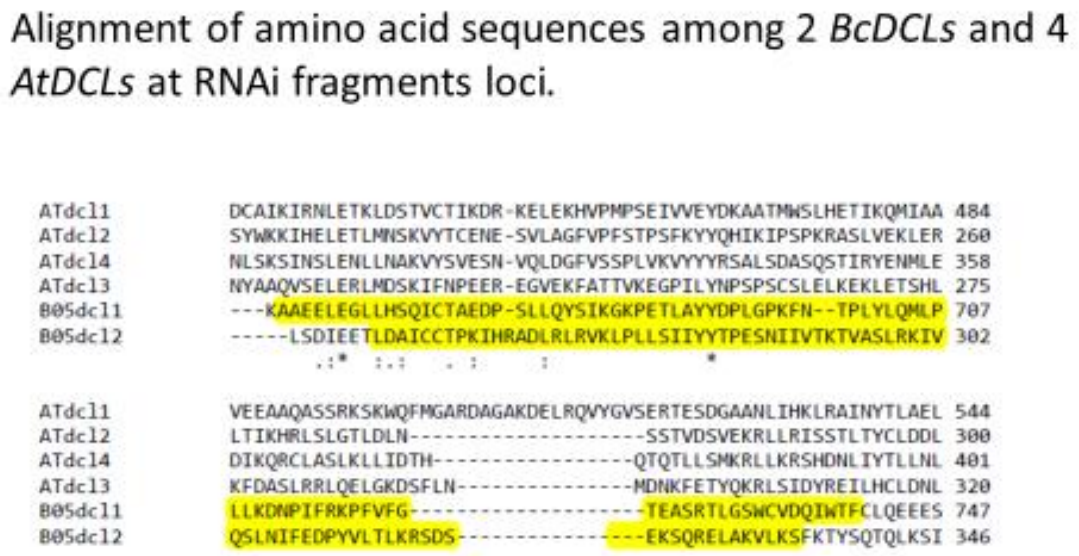


Figure 3.4 HIGS of BcDCLs in Arabidopsis enhances plant resistance to *B. cinerea*

A) The expression level of siRBcDCLs from wild type and three selected transgenic lines transformed with HIGS-BcDCLs as measured by Northern blot. U6 was used as loading control. B) Three selected HIGS-BcDCL lines as well as the wild type plants were infected with *B. cinerea* using drop inoculation. The pictures were taken after 4 dpi. Three biological repeats indicated similar results.

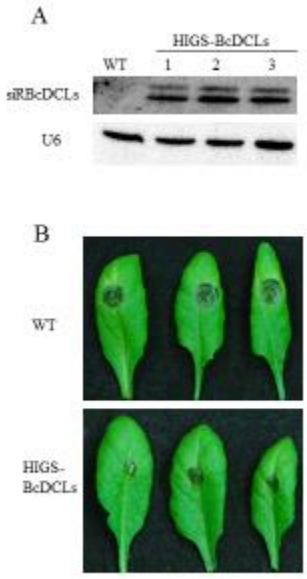


Figure 3.5 VIGS of BcDCLs in tomato enhances plant resistance to *B. cinerea*

A) The fifth, sixth, and seventh leaves of tomato VIGS-RB and VIGS-BcDCLs plants were detached and infected with *B. cinerea* using spray inoculation. Pictures were taken 3 dpi. Three biological repeats indicated similar results.

B) The levels of siRBcDCLs from the corresponding infected leaves were measured by Northern blot. U6 was used as loading control.

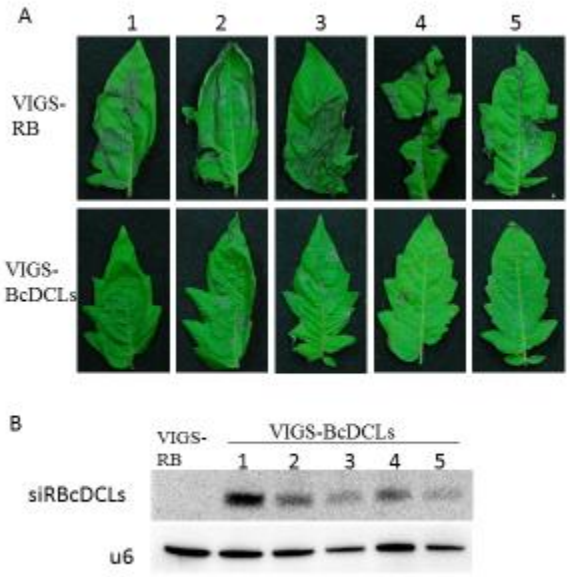


Table 3.1 Bc-sRNA reads numbers in *B. cinerea* WT and *dcl1 dcl2* double mutant libraries

Class	*dcl1dcl2*	WT
Retrotransposon	**11,763**	**309,798**
ORF	4,598,709	1,211,701
Intergenic region	3,505,618	1,348,004
tRNA	13,800,737	2,897,184
rRNA	**14,920,307**	**42,372,765**
Total reads	36,837,134	59,236,120

Conclusions and Perspectives

During the last 10 years, the role of host plant sRNAs in host-pathogen interactions has been extensively investigated. Both plant miRNAs and siRNAs contribute significantly to plant defense responses against pathogens. For example, miRNA directed-*R* gene expression is conserved among various plant species. Pathogen infection activates the expression of *R* genes via inhibition of corresponding miRNA accumulation. However, whether the pathogen sRNAs are also involved in the host-pathogen interactions is not known.

In my thesis, we demonstrated that sRNAs of fungal pathogen *B. cinerea* could also act as a novel type of effector molecule. Three Bc-sRNAs, Bc-siR3.1, Bc-siR3.2 and Bc-siR5, were identified and characterized. These sRNA effectors were induced during pathogen infection processes, leading to the down regulation of the host target genes. We further verified that these sRNA effectors hijacked host AGO1 protein and suppressed host immunity related target genes. We generated *B. cinerea dcl1 dcl2* double mutant strain and it failed to produce these sRNA effectors, leading to compromised virulence on host plants.

In addition, we also explored another Bc-sRNA effector, Bc-siR37, which has multiple candidate target genes in both Arabidopsis and tomato. Most of these target genes are putative plant immunity genes. Three predicted Arabidopsis target genes, *Atwrky7*, *Atpmr6* and *Atfei2*, were confirmed to be the target genes of Bc-siR37, and they all play a significant role in the defense responses against *B. cinerea*. Thus, we proposed that our

predicted host target genes of the Bc-sRNA effectors could help to quickly identify the plant immunity genes against *B. cinerea*.

Moreover, we found that the majority of the retrotransposon derived Bc-sRNAs were DCL-dependent. Interestingly, most of the predicted sRNA effectors were generated from LTR-retrotransposon regions, and almost all of them were absent in the *B. cinerea dcl1 dcl2* double mutant. Thus, *B. cinerea* DCL1 and DCL2 contribute to its virulence by producing many of the Bc-sRNA effectors. Based on this information, I applied HIGS and VIGS against *B. cinerea dcl1 dcl2* in Arabidopsis and tomato plants, respectively, and successfully enhanced plant immunity against *B. cinerea*.

There are still plenty of questions regarding this topic that remain to be addressed in the future. For example, as sRNA and RNAi are conserved among most eukaryotes, including the eukaryotic pathogens, it is worth to know whether other eukaryotic pathogens also use sRNAs as effectors to strengthen their virulence. Although it is clear that Bc-sRNAs are secreted from pathogens to host cells during infection processes, how these sRNAs are protected and delivered still remains to be clarified. Since retrotransposon regions are hot spots for Bc-sRNA effector production, further experiments are needed to verify how these retrotransposons contribute to *B. cinerea* pathogenicity. HIGS exemplifies the movement of artificially expressed silencing triggers from host to the pathogen so far, but whether this process can occurred naturally is still unknown. During HIGS, the host is introduced with hairpin RNAs or dsRNAs, which can be digested to sRNAs by the host RNAi machinery. Thus, it is not clear whether it is the dsRNAs, sRNAs, or both that can act as mobile RNAs to regulate pathogen genes. Since most pathogen

protein effector molecules are recognized by the host proteins to trigger race-specific defense responses in the host plants, it is likely that the host plants also evolved sensor proteins to recognize pathogen sRNA effectors and probably also induce host immunity against the pathogens. A better understanding of the mechanisms behind these host plant and pathogen interactions will aid in the development of novel plant disease management methods.