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Small RNAs in Plant Immunity and Virulence of Filamentous Pathogens

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Keywords

RNA interference, gene silencing, host–pathogen arms race, nucleotide-binding leucine-rich repeat receptor, pentatricopeptide repeat protein, small RNA trafficking

Abstract

Gene silencing guided by small RNAs governs a broad range of cellular processes in eukaryotes. Small RNAs are important components of plant immunity because they contribute to pathogen-triggered transcription reprogramming and directly target pathogen RNAs. Recent research suggests that silencing of pathogen genes by plant small RNAs occurs not only during viral infection but also in nonviral pathogens through a process termed host-induced gene silencing, which involves *trans*-species small RNA trafficking. Similarly, small RNAs are also produced by eukaryotic pathogens and regulate virulence. This review summarizes the small RNA pathways in both plants and filamentous pathogens, including fungi and oomycetes, and discusses their role in host–pathogen interactions. We highlight secondary

small interfering RNAs of plants as regulators of immune receptor gene expression and executors of host-induced gene silencing in invading pathogens. The current status and prospects of *trans*-species gene silencing at the host–pathogen interface are discussed.

INTRODUCTION

RNA interference (RNAi) by small RNAs (sRNAs) has a broad impact on cellular processes in eukaryotes (50). Based on sequence complementarity, sRNAs regulate their target gene(s) by transcription repression, transcript cleavage, or translation inhibition. sRNA-mediated silencing is quantitative, making it particularly suitable for fine-tuning gene expression (4, 6). Furthermore, sRNAs can be mobile, enabling a systemic response to environmental signals as well as target gene manipulation in organisms engaged in an intimate symbiosis (61, 85, 109). These features place sRNA-mediated regulation in a uniquely important position during plant–pathogen interactions with the potential of conferring precise temporal and spatial controls.

Constantly challenged by potential microbial pathogens in the surrounding environment, plants have evolved a sophisticated and robust immune system. Pattern-triggered immunity (PTI) relies on the recognition of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (18, 150). Classic responses triggered by the activation of PTI include reactive oxygen species (ROS) production, cell wall reinforcement, and antimicrobial compound secretion (150). Among the antimicrobial agents are specific sRNAs, which have recently been shown to be enriched in the extracellular space (8) and to silence target genes in invading filamentous pathogens (19, 58, 145). Although PTI prevents the colonization of the majority of potential pathogens, successful pathogens are able to defeat this line of defense through activities of virulence proteins called effectors (38). In particular, filamentous pathogens produce suppressors of RNA silencing, illustrating an arms race centered on sRNA-mediated defense (58, 102, 137, 139). As a counteractive strategy, plants evolved another layer of defense that depends on the recognition of pathogen effectors by the nucleotide-binding leucine-rich repeat (NLR) receptors (30, 67). Activation of the NLR immune receptors results in effector-triggered immunity (ETI), which often associates with programmed cell death to restrict the spread of the pathogen (33). However, the *NLR* genes must be precisely regulated to prevent fitness cost and detrimental autoimmunity. One of the mechanisms that keep the *NLR*s in check is gene silencing. In some plants, sRNAs potentially regulate *NLR* genes in a global manner (21, 47, 143).

As a ubiquitous gene regulation mechanism in eukaryotes, sRNAs are also produced by eukaryotic pathogens to regulate virulence. Many fungal and oomycete pathogens have been analyzed for sRNA profiles, and gene silencing has been implicated in regulating the virulence of some of these pathogens. For example, silencing of effector genes is a mechanism utilized by the oomycete pathogens *Phytophthora* to evade host recognition (99, 103). In addition, pathogen-derived sRNAs have also been shown to affect host gene expression as a virulence mechanism (40, 124). A potential bidirectional, *trans*-species trafficking of sRNAs during plant–pathogen interaction is an active area of research (61).

Here, we summarize the increasing body of evidence indicating the mechanisms by which sRNAs influence disease development through endogenous and *trans*-species gene silencing. Gaps of knowledge and future perspectives are also discussed. We focus on nonviral diseases caused by filamentous pathogens, including fungi and oomycetes. For the critical role of RNA silencing in antiviral immunity, please refer to a recent review (56).

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SMALL RNA REGULATION OF PLANT IMMUNITY

Plant Small RNAs: Classification, Biogenesis, and Function

Plant sRNAs are generally 20–24 nucleotides (nt) in length and regulate gene expression at the transcriptional and posttranscriptional levels (16, 17). sRNAs can mediate transcriptional gene silencing (TGS) through RNA-directed DNA methylation or histone methylation (89); they can also trigger target degradation or translation repression by pairing to mRNAs, thus mediating posttranscriptional gene silencing (PTGS) (107).

Plant sRNAs can be classified into two major categories, microRNAs (miRNAs) and small interfering RNAs (siRNAs), based on differences in precursor sources, biosynthetic pathways, and molecular functions (16). miRNAs are generated from RNA polymerase II transcribed single-stranded precursors, which fold into imperfectly paired stem-loop structures. These precursors are then processed by the RNase III–type ribonuclease Dicer-like 1 (DCL1) to produce miRNA/miRNA* duplexes, which are subsequently methylated by HUA ENHANCER1 (HEN1) to protect the 3′ terminal nucleotide from 3′→5′ degradation (64, 106). One strand of the duplex, also called the miRNA strand, is loaded into ARGONAUTE (AGO) proteins, which form RNA-induced silencing complexes (RISC) and carry out the target gene–silencing functions at the PTGS level (**Figure 1**) (106).

Compared to miRNAs, siRNAs are derived from perfectly matched complementary double-stranded RNAs (dsRNAs), often the products of RNA-dependent RNA polymerases (RDRs) (16). Production of siRNAs can be triggered by exogenous RNAs such as transgenes or viruses, but there are also numerous siRNAs whose biogenesis requires the cleavage of specific endogenous transcripts guided by a subset of miRNAs and the subsequent RDR6-dependent synthesis of dsRNA precursors (16). The dsRNAs are then processed by DCL3 or DCL4 homologs to produce an array of siRNAs that are often spaced at 21- or 24-nt phased intervals. As such, these secondary siRNAs are also called phased siRNAs (phasiRNAs) (27). For example, the plant-specific, 21-nt *trans*-acting siRNAs (tasiRNAs) are generated following the miRNA-guided cleavage of the noncoding *TAS* transcripts (2, 49). Transcripts of coding genes, especially *NLR* and pentatricopeptide repeat (*PPR*) genes, can also spawn 21-nt secondary siRNAs (46). Similar to miRNAs, secondary siRNAs are methylated by HEN1 and subsequently loaded into the AGO complexes to induce targeted gene silencing (**Figure 1**) (17, 64). The functional significance of the secondary siRNA pathway lies in potential widespread silencing through a regulatory cascade in which genes can be silenced by both the primary miRNA and the siRNAs. Importantly, secondary siRNAs could also target genes that are not regulated by the primary miRNAs (46), expanding the impact of gene silencing.

miRNA Regulation of Defense/Growth Tradeoff

Activation of the defense response usually comes with a cost to growth (62, 71). Because miRNAs are key regulators of plant growth and development, activation of plant immunity is often correlated with upregulation of growth-regulating miRNAs upon pathogen perception. These pathogen-responsive miRNAs act as fine-tuners to balance the defense/growth tradeoff by mainly influencing the phytohormone signaling pathways (76, 87, 96).

Several conserved miRNAs are key regulators of auxin signaling, which has an antagonistic interaction with the salicylic acid (SA)-mediated defense against pathogens feeding on living host cells (called biotrophs or hemibiotrophs) (51). For example, miR393, which targets the TIR1/AFB (transport inhibitor response 1/auxin-related F box) auxin co-receptors, is induced by MAMP/PAMP treatments (83, 96, 129). Reduced accumulation of miR393 in soybean led to

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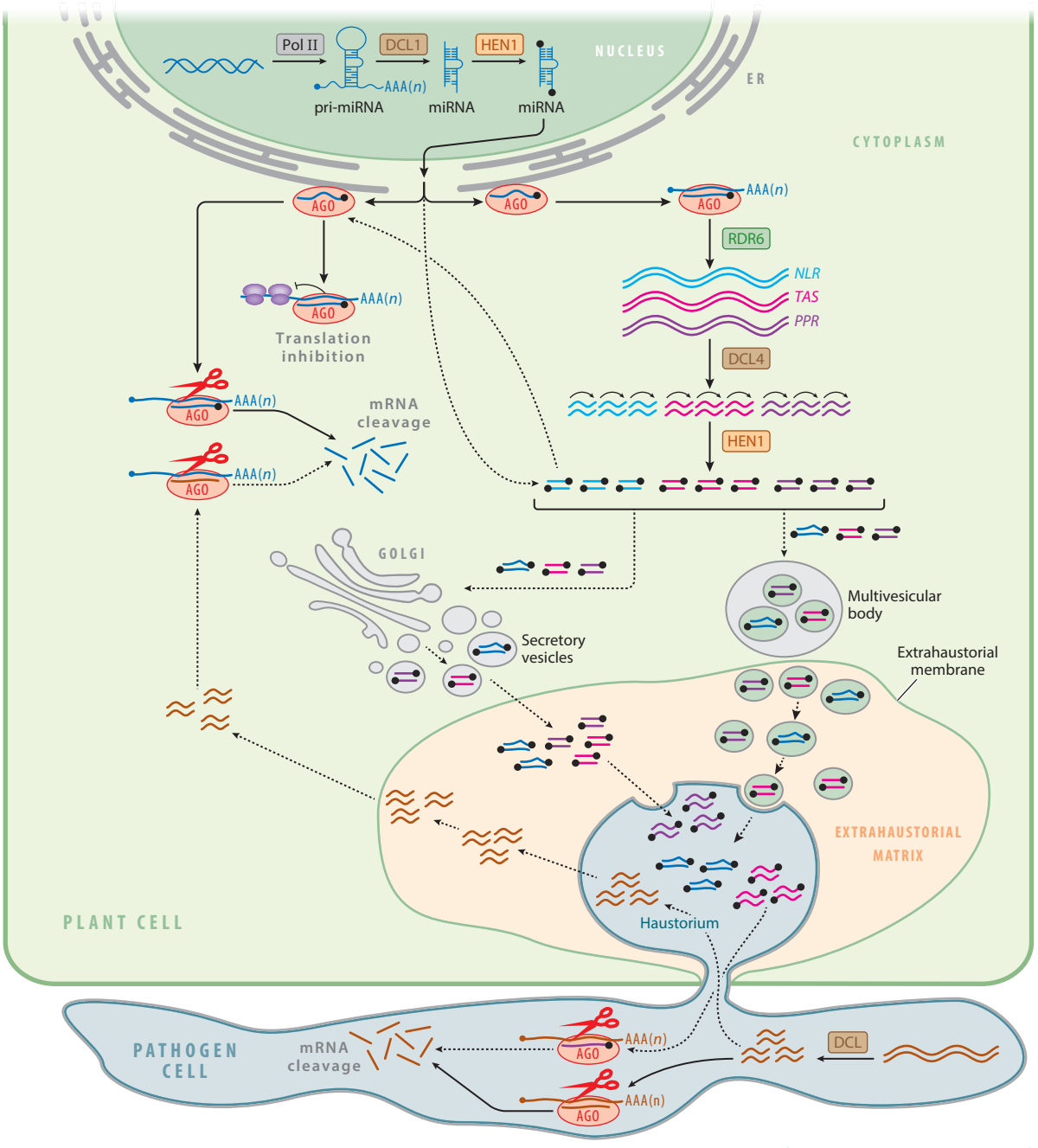

(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Small RNA (sRNA)–mediated gene silencing in plant–pathogen interactions. Plants produce two classes of sRNAs: microRNA (miRNA) and small interfering RNA (siRNA). miRNAs are processed by DCL1 from the precursor transcripts called pri-miRNA, and siRNAs are produced using long double-stranded RNAs as precursors via the RDR6/DCL4 pathway. Major siRNA-producing transcripts include *NLR*, *TAS*, and *PPR*. Both miRNAs and siRNAs are methylated by HEN1 and regulate target gene expression by transcript cleavage and translation inhibition. During infection, biotrophic and hemibiotrophic filamentous pathogens form specialized infection structures, haustoria, at the host–pathogen interaction interface. The plant cell and the pathogen cell are separated by the extrahaustorial matrix (EHMx). Putative secretion pathways of sRNAs from the plant cell to the EHMx include the extracellular vesicle (EV)-dependent route and EV-independent route that may involve secretary vesicles. Selected miRNAs could also be secreted through either or both routes. *TAS1/2*- and *PPR*-derived secondary siRNAs have been implicated in silencing target genes in invading fungal and oomycete pathogens. Some pathogens also secrete sRNAs to facilitate disease development by targeting host genes. So far, HEN1 orthologs are not found in filamentous pathogens, and pathogen-produced sRNAs are presumably unmethylated. Dashed lines represent events that require further experimental support. Abbreviations: AGO, Argonaute; DCL, Dicer-like protein; NLR, nucleotide-binding leucine-rich repeat; PPR, pentatricopeptide repeat protein; pri-miRNA, primary microRNA.

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hypersusceptibility to *Phytophthora sojae*, suggesting that it is a positive regulator of plant defense (129). Several other auxin-regulating miRNAs, such as miR160, miR164, and miR167, are also upregulated in plants infected with filamentous pathogens (76). Increased levels of these miRNAs repress auxin signaling, contributing to a switch from growth to defense.

miRNA Regulation of Immune Receptors

Receptors are key components of the plant immune system. The membrane-embedded receptor-like kinases or receptor-like proteins sense non-self molecular signatures, leading to PTI, and the intracellular NLR receptors recognize pathogen effectors and activate ETI (150). However, inappropriate expression or activation of immune receptors, especially NLRs, can cause a general reduction in fitness and detrimental autoimmunity (71, 80, 116). sRNAs are implicated in controlling the expression of NLRs during different developmental stages (35) and in the absence of infection. *NLR* genes are common targets of miRNAs in a broad range of plants (47, 79, 111, 146). NLR proteins can be further classified into Toll interleukin 1 receptor (TIR)-NLRs (or TNLs) and coiled-coil (CC)-NLRs (or CNLs) based on distinctive N-terminal motifs. Both TNLs and CNLs can be regulated by miRNAs. The conserved, but also unusually diverse, miR482/2118 superfamily targets *NLR* genes by binding to the coding sequence of the highly conserved P-loop motif (111). Decreased accumulation of miR482/2118 family members was observed in soybean infected with *P. sojae* (55, 129) or the fungal pathogen *Phakopsora pachyrhizi* (77) as well as in cotton infected with the fungal pathogen *Verticillium dahliae* (135). Downregulation of these miRNAs presumably contributes to pathogen-inducible expression of NLRs. Importantly, attenuation of miR482/2118 functions in tomato led to enhanced resistance to *Phytophthora infestans* and the bacterial pathogen *Pseudomonas syringae*, demonstrating the biological significance of miRNA-mediated regulation of NLRs (21).

In addition to the direct silencing of targeted *NLR* transcripts, miR482/2118 triggers the production of secondary siRNAs, thereby having the potential to suppress an amplified number of *NLR*s in *trans* or in *cis* (21, 47, 111). Other miRNAs that target *NLR* genes, such as miR6019 in tobacco (79) and miR9863 in barley (84), also exert their functions through this dual mode of action, indicating an important role of secondary siRNA production in NLR regulation. Engaged in a constant coevolutionary arms race, NLRs represent large gene families with a high degree of diversification in plant genomes. Gene silencing that depends on miRNAs may not be sufficiently adapted to the fast evolution of NLRs, making secondary siRNA-based regulation an attractive strategy. Indeed, a search for siRNAs in *Medicago truncatula* found hundreds of siRNA-generating loci, the majority of which (79 out of 112) were *NLR* genes (45). Remarkably, three abundant

miRNA families, miR1507, miR2109, and miR2118, trigger the production of secondary siRNAs from 74 of the 79 siRNA-spawning *NLR* transcripts. These miRNAs target the conserved domains in NLRs: miR1507 has complementarity to sequences in *CNL* genes that encode the kinase-2 motif; miR2109 targets the TIR-1 motif in TNLs; and miR2118 targets sequences encoding the conserved P-loop in both TNLs and CNLs (143). As such, it was hypothesized that control of a large number of NLRs could be accomplished by a few miRNAs. However, a global impact of miRNAs on the *NLR* transcriptome through secondary siRNA production has not been experimentally confirmed, even though a minor but overall reduction of *NLR* transcript levels was observed to influence the defense response of tomato in a quantitative manner (21). Another possibility is that these secondary siRNAs may regulate NLR protein accumulations through translation inhibition. Further investigations are needed to determine the underlying regulatory mechanism and functional impact of secondary siRNAs on global NLR levels.

HOST-INDUCED GENE SILENCING IN INVADING FILAMENTOUS PATHOGENS

The non-cell-autonomous feature of RNA silencing suggests sRNAs can be mobile molecules that move cell-to-cell and silence a target gene(s) in the recipient cells. The motility of many siRNAs and some miRNAs within plants has been well documented (85). More recent research indicates that sRNAs may also traffic from plants to pathogens in a process termed host-induced gene silencing (HIGS) (10). The first observations of HIGS were made when specific gene transcripts showed reduced accumulation in nematodes and insects when they fed on transgenic plants that produced artificial RNAi constructs (11, 60). To date, examples of the successful application of HIGS have been expanded to control a broad range of pathogens, including parasitic plants, fungi, and oomycetes in various plant species (e.g., 52, 63, 97, 144). Importantly, specific plant sRNAs have recently been discovered to silence pathogen genes during natural infection as a native defense mechanism (19, 58, 145).

Two Plant miRNAs Target Virulence Genes in the Fungal Pathogen *Verticillium dahliae*

The first example of HIGS as a natural defense mechanism in nonviral pathogens was reported in cotton, where two miRNAs, miR159 and miR166, were found to silence virulence-related genes in the fungal pathogen *V. dahliae* (145). Both miR159 and miR166 were induced upon infection, and their sequences show complementarity to *V. dahliae* genes encoding a Ca^{2+}-dependent cysteine protease (Clp-1) and an isotrichodermin C-15 hydroxylase (HiC-15), respectively. Transcript levels of *Clp-1* and *HiC-15* were reduced in fungal hyphae recovered from infected cotton tissues, and miR159 and miR166 were also detected from the hyphae. Mutants of *V. dahliae* with *Clp-1* or *HiC-15* knocked out displayed reduced virulence, consistent with the hypothesis that miR159 and miR166 produced by the infected cotton plants entered the invading fungal cells and triggered cleavage of targeted pathogen transcripts that are required for virulence (144, 145).

This research provided strong evidence that plant-derived miRNAs were present in the fungal cells and available for gene silencing. miR159 and miR166 could not only be detected in *V. dahliae* cultured from hyphae recovered from infected cotton tissues but also remained detectable in the progenies of the fungal culture, suggesting a high level of stability in the pathogen cells. miR159 and miR166 are among the most abundant miRNAs in cotton and *Arabidopsis thaliana* (145). Whether the high abundances of these miRNAs are related to their movement and stability in the pathogen is an interesting question. In addition to targeting pathogen genes, miR159 regulates vegetative phase changes in plants (3) and miR166 is involved in root development (23). Both

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miRNAs are conserved in plants with conserved endogenous targets and functions. It would be interesting to monitor the coevolutionary dynamics of these miRNAs with their pathogen targets.

Secondary siRNAs Enhance Plant Immunity Through Host-Induced Gene Silencing

Following this initial report of natural HIGS by miRNAs, studies in *A. thaliana* uncovered a role of secondary siRNAs in silencing pathogen genes during infection. Two tasiRNAs, derived from the noncoding *TAS1* and *TAS2* transcripts, respectively, were shown to silence target genes in the fungal pathogen *Botrytis cinerea*. Deletion of the tasiRNA-targeted genes in *B. cinerea* led to reduced virulence activity, suggesting that they contribute to disease development (19). In a separate study, a pool of secondary siRNAs derived from a subset of *PPR* gene transcripts can potentially target multiple genes in the oomycete pathogen *Phytophthora capsici*. One of the target genes encodes a U2-associated splicing factor and is required for sporulation and the full virulence of *P. capsici* (58). These findings are consistent with the hypersusceptibility phenotype of the *rdr6* mutant of *A. thaliana*, which is abrogated in secondary siRNA production, to both pathogens.

Further support for the biological significance of the secondary siRNA pathway in plant immunity comes from the discovery that filamentous pathogens encode effectors to suppress this pathway in plants (102, 137): The *Phytophthora* effector PSR2 specifically affects siRNAs produced from *TAS1*, *TAS2*, and *PPR* transcripts in *A. thaliana* (58), and the effector PgtSR1 of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* also reduces the production of secondary siRNAs (139). Research on effector functions has facilitated the identification of novel host immune components and mechanisms. The virulence activities of PSR2 and PgtSR1 are consistent with a role of secondary siRNAs in plant defense during fungal and *Phytophthora* infection. It is noteworthy that the *rdr6* mutant of *A. thaliana* is also hypersusceptible to *V. dahliae* (42), raising the possibility that secondary siRNAs may contribute to plant defense against a broad range of pathogens.

HIGS requires sRNAs to traffic from the host to the invading pathogen. Differences in the biosynthetic pathways of miRNAs and siRNAs may affect their mobility. When the miRNAs and tasiRNAs were designed to have identical sequences, tasiRNA-based silencing spread into a broader area than miRNA-based silencing, indicating that secondary siRNAs may be more mobile and robust in non-cell-autonomous silencing (34). Furthermore, analysis of apoplastic sRNAs in *A. thaliana* revealed a striking extracellular accumulation of secondary siRNAs, including *TAS*-, *NLR*- and *PPR*-siRNAs, suggesting that they are specifically secreted by plant cells (8). Many miRNAs are less mobile: The sites of pri-miRNA transcription and miRNA function were found to be well correlated (4, 100). tasiRNAs and *PPR*-siRNAs were also detected from extracellular vesicles (EVs) (19, 58). These findings point to the possibility that secondary siRNAs may be a major contributor of HIGS.

A Role of Pentatricopeptide Repeat Gene-Derived Secondary siRNAs in Host-Induced Gene Silencing

In eudicots, *PPR* genes are one of the main sources of secondary siRNA production (130). PPRs constitute a large gene family in plants, with more than 400 members in most eudicots. PPR proteins can be categorized into subclasses based on the nature of their PPR motifs (9). Most functionally characterized PPR proteins have sequence-specific nucleotide-binding activities and function as molecular adaptors that direct RNA processing complexes to target transcripts in mitochondria and chloroplasts (9). A subset of *PPR* genes is recruited to the miRNA-triggered

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Table 1 siRNA production from pentatricopeptide repeat genes and their trigger miRNAs in various plant species

Plant family	Species	miRNA trigger	Putative mechanism	Reference(s)
Araliaceae	*Panax notoginseng*	miR1509	*TAS* mediation	25
Brassicaceae	*Arabidopsis thaliana*	miR173, miR161	Direct targeting or *TAS* mediation	59
	Capsella rubella	miR173, miR161		113
Euphorbiaceae	*Manihot esculenta*	NA	NA	73
Fabaceae	*Glycine max*	miR1508, miR1509	Direct targeting or *TAS* mediation	130
	Medicago truncatula	miR1509	*TAS* mediation	
Pinaceae	*Picea abies*	miR11425a	Direct targeting	131
Rosaceae	*Malus* × *domestica*	miR7122	*TAS* mediation	130
	Prunus persica	miR7122	Direct targeting or *TAS* mediation	
	Fragaria vesca	fve-PPRtri1/2		
Salicaceae	*Populus trichocarpa*	miR6427	Direct targeting	130
Sapindaceae	*Litchi chinensis*	NA	NA	88
Solanaceae	*Nicotiana tabacum*	miR7122	*TAS* mediation	130
Rutaceae	*Citrus sinensis*	miRC1	Direct targeting	130, 148
Vitaceae	*Vitis vinifera*	miR7122	Direct targeting or *TAS* mediation	130

Abbreviation: NA, not available.

secondary siRNA-generating pathway. For instance, the vast majority of *PPR*-siRNAs in *A. thaliana* are produced from 17 *PPR* genes clustered in chromosome 1 (59).

*NLR*s are targeted by miRNA triggers at the highly conserved regions, leading to miRNA-mediated regulation and subsequent siRNA generation from a large number of genes (132, 143). This was proposed to be an efficient way to maintain the overall low expression level of genes belonging to the same gene family (46). However, *PPR*s seem to be different. Although PPR is one of the largest gene families associated with sRNAs, only a small set generates siRNAs through a more complicated mechanism (59). The miR7122 superfamily, containing evolutionarily related miRNAs, directs the generation of siRNAs from a limited number of *PPR* transcripts in a wide range of plant species (130). Production of siRNAs can be triggered both by direct targeting of miRNAs, i.e., via the miRNA-*PPR* circuit, and through the mediation of noncoding *TAS* genes, i.e., via the miRNA-*TAS*-*PPR* circuit (130). In *A. thaliana*, *PPR* transcripts are directly targeted by miR161 and indirectly regulated by *PPR*-targeting tasiRNAs generated by the miR173-*TAS1/2* module (59, 130). In legumes, miR1509 relies on two layers of *TAS* actions to incite siRNA production from *PPR* transcripts (130). In addition to the miR7122 family members, new and species-specific miRNAs have also evolved to direct siRNA production from *PPR*s (**Table 1**). Interestingly, similar to miR7122, these miRNAs trigger *PPR*-siRNA generation via direct targeting, *TAS*-mediated targeting, or both. The complexity in the miRNA-(*TAS*)-*PPR* transcripts-siRNA routes potentially provides multiple checkpoints to fine-tune the accumulation of *PPR*-siRNAs as a highly conserved process in dicots, hence indicating a functional importance of these siRNAs.

PPR proteins are characterized by 2–30 tandem repeats of ~35 amino acid peptides (9). Because the PPR motifs are often degenerated, *PPR* genes share low sequence identity at the nucleotide level. Intriguingly, the sequences of *PPR* genes that generate siRNAs exhibit a unique

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pattern of intraspecific expansion and diversification (59, 91), a feature that could be explained by engagement in a coevolutionary arms race with pathogens. On one hand, the fast evolution of siRNA-producing *PPR* genes would drive the rapid evolution of the sRNA triggers, which is indeed observed in the sequence divergent miR7122 family and *PPR*-targeting tasiRNAs. On the other hand, it would also lead to the generation of siRNAs with diverse sequences; this is consistent with a proposed shotgun mechanism that involves a pool of sRNAs and the potential of targeting multiple genes in various pathogens (57, 58, 61). In addition, the remarkable intraspecific diversification may also reflect species-specific gene silencing in adapted pathogens. Future research is warranted to examine how widespread the role of *PPR*-siRNAs is in host–pathogen interactions and how this novel defense mechanism can be incorporated into breeding programs to enhance disease resistance.

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SMALL RNA PRODUCED BY FILAMENTOUS PATHOGENS

sRNAs are widely produced by eukaryotes, including fungal and oomycete pathogens. Target prediction indicates that sRNAs from these pathogens may regulate both endogenous genes and potential host targets during infection. However, the RNAi pathway has been characterized in only a handful of model organisms, and very few sRNAs have been studied experimentally for their biogenesis and function (**Table 2**). This is in part due to technical challenges to generate mutants in these organisms, which also utilize diverse sRNA biosynthetic pathways. Here, we summarize the core components of the RNAi pathway and sRNA profile of representative fungal pathogens and the oomycete pathogen *Phytophthora* and discuss their potential role in regulating virulence. It is important to note that these pathogens have different infection styles ranging from biotroph (feeding from living host cells throughout its life cycle) or necrotroph (feeding from killed host cells) to hemibiotroph (two-phased infection with an initial biotrophic stage and a subsequent necrotrophic stage) (51). Therefore, the virulence strategies and mechanisms utilized by these pathogens are also different.

Small RNAs from Phytopathogenic Fungi

The fungal kingdom exhibits enormous diversity in ecology, morphology, and lifestyle. It has been suggested that a patrimonial RNAi pathway composed of RDRs, DCLs, and AGOs has undergone numerous adaptations, leading to gene losses as well as evolution of novel sRNA biogenesis mechanisms in various fungal species (13, 24). For example, the core components of the RNAi machinery are absent from some fungal species, including *Ustilago maydis*, a biotrophic pathogen that causes the smut disease of maize (48, 95). In addition, the model filamentous fungus *Neurospora crassa* produces distinct sRNA populations through at least four different biosynthetic pathways that utilize different combinations of enzymes (78). Similar to plants and animals, fungal sRNAs contribute to antiviral immunity (20, 93) and maintenance of genomic stability and integrity (13). Below, we summarize phytopathogenic fungal species from which the sRNA pathway has been functionally characterized in pathogenesis (**Figure 2**).

***Magnaporthe oryzae*.** The hemibiotrophic ascomycete *M. oryzae* is the causal agent of rice blast, one of the most devastating diseases of cultivated rice (128). *M. oryzae* encodes two DCLs, three AGOs, and three RDRs (69, 105). Gene silencing by sRNAs was first demonstrated by using a hairpin RNA of *GFP*, which triggered the accumulation of 19–23-nt siRNAs that lead to *GFP* silencing (68). *Mo*DCL2, but not *Mo*DCL1, is required for the production of the *GFP*-siRNAs (69). A comprehensive analysis of mutants of the RNAi core components revealed a key role of sRNAs in regulating conidia development. In addition, *mordr1* and *moago3* mutants also showed

Table 2 Summary of small RNAs identified in plant filamentous pathogens that have validated targets and potential functions

Pathogens	Small RNA	Length (nt)	Mature sequences	Predicted target	Putative function	Reference(s)
Botrytis cinerea	*Bc*-siR3.1	21	CGGGUGGAUGUUCUAGGUGUU	*AtMPK1/2*	Silence host genes	122, 124
	Bc-siR3.2	21	UGGAUGUUCUAGGUGUUACAU	*AtPRXIIF*		
	Bc-siR5	21	UUCAUAUGUAAGGCUCAGUUU	*AtWAK*		
	Bc-siR37	21	UAGUAGUAGUAGAAGGAGGAA	*AtWRKY7/AtFEI2*		
Cordyceps militaris	*Cm*-milR4	19	AGUCCGACGACGAGGAGCC	ND	Sexual development	110
	Cm-milR16	21	AGUAGUUGGGUCGGUGACGAC	ND		
Fusarium graminearum	*Fg*-milR2	22	UAGGAAAGGCAGUUAACUAGGA	*FgbioH1*	Biotin biosynthesis	54, 114
	Fg-ex-siRNA1	24	UCACGCGUUGGGACUUGGGUCCUU	*FgSG_10502*	Unknown	
	Fg-ex-siRNA2	25	AAAGAGACUCGACCUCUGGCGCCCU	*FgSG_03222*	Unknown	
Magnaporthe oryzae	*Mo*-milR146-3	20	AGCGAGCGCUGCAGUGCCUG	ND	Appressorium formation and virulence	82
	Mo-milR236-5p	20	UGUCUGGAUCGAUACGGAUG	*MoHAT1*		
	Mo-milR293-5p	20	CCAUGGGAACUGUUUCUGGU	ND		
Penicillium italicum	*Pit*-milR7	22	UGGCUGGAGCAUGCGCUUGAUU	*PitAP2/B3* transcription factor	Unknown	140
Puccinia striiformis f. sp. *tritici*	*Pst*-milR1	20	GGAAGGAAAUGGUGAGGAAU	*TaPR2*	Silence a host gene	120
Sclerotinia sclerotiorum	*Ss*-milR1	22	UCUCGAGCCGUAUAGACAGUGU	ND	Sclerotial development	149
	Ss-milRC2	21	GGUGUUGUGGUUUAGUGGUAU	ND		
Verticillium dahliae	*Vd*-milR1	21	AUUGGUGAGCUGAUUAGCCUU	*VdHY1*	Virulence	66
Valsa mali	*Vm*-milR16	19	GUUUCGGGUUGCACGGGUU	*VmSNF1*, *VmDODA*, and *VmHY1*	Virulence	133
Hyaloperonospora arabidopsidis	*Hpa*-sRNA2	21	CCUCAGGGCUCUUUAAUUUCU	*AtWNK2*	Silence host genes	40
	Hpa-sRNA30	21	GUCUAAAUCCUUUACCUUUAA	*AtWNK5*		
	Hpa-sRNA90	21	CGAUCACAAGUACUAGUAUUU	*AtAED3*		
Phytophthora infestans	*Pi*-miR8788	21	CUACCAAGCGUACCAGCGCCG	*PiAAAP*	Unknown	44
Phytophthora sojae	*Ps*-miR8788	22	CUACCAAGCGUACCAAUACCGU	*PsAAAP*	Unknown	44
Phytophthora ramorum	*Pr*-miR8788	21	CUACCAAGCGUACCAACGCCG	*PrAAAP*	Unknown	44

Abbreviations: *AtAED3*, *Arabidopsis thaliana* apoplastic, enhanced disease susceptibility1-dependent 3; *AtMPK1/2*, *Arabidopsis thaliana* mitogen-activated protein kinase 1/2; *AtPRXIIF*, *Arabidopsis thaliana* peroxiredoxin IIF; *AtWAK*, *Arabidopsis thaliana* cell wall-associated kinase; *AtWNK2/5*, *Arabidopsis thaliana* with no lysine (K) kinase 2/5; *AtWRKY7*, *Arabidopsis thaliana* WRKY DNA-binding protein 7; *FgbioH1*, *Fusarium graminearum* pimeloyl-ACP methyl ester carboxylesterase; *MoHAT1*; *Magnaporthe oryzae* histone acetyltransferase; ND, not determined; *PiAAAP*, *Phytophthora infestans* amino acid/auxin permease; *PitAP2/B3*, *Penicillium italicum* AP2/B3-like; *TaPR2*, *Triticum aestivum* pathogenesis-related protein 2; *VdHY1*, *Verticillium dahliae* hypothetical protein 1; *VmDODA*, *Valsa mali* 4,5-DOPA dioxygenase extradiol; *VmHY1*, *Valsa mali* hypothetical protein 1; *VmSNF1*, *Valsa mali* sucrose nonfermenting 1.

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reduced hyphae growth in vitro and decreased virulence activity in rice. Whether the impact of these mutants on virulence activity was attributed to growth deficiency is unknown (105).

Profiling the endogenous sRNAs revealed 20–21-nt miRNA-like sRNAs (milRNAs), long terminal repeat (LTR) retrotransposon-siRNAs (LTR-siRNAs), and methylguanosine-capped and polyadenylated sRNAs (CPA-sRNAs) in *M. oryzae* (53, 98). sRNAs of 19–23 nt are enriched from intergenic regions and repetitive elements in mycelia; however, a greater proportion of 28–35-nt sRNAs mapped to tRNA loci, protein-coding genes, and LTR retrotransposons was found in

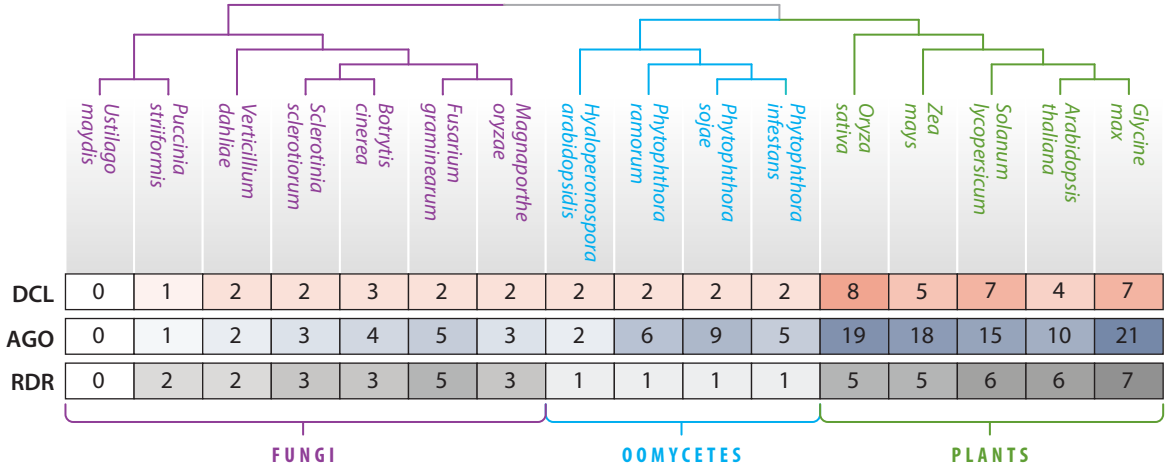

Figure 2

A summary of core enzymes in the RNA silencing pathway of representative fungal pathogens, oomycetes, and plants. The phylogenetic tree was generated using the amino acid sequences of β-tubulin by RAxML (randomized axelerated maximum likelihood) and the model PROTGAMMALG. None of the pathogenic fungal or oomycete species encode orthologs of the methyltransferase HUA-ENHANCER 1 (HEN1), which is highly conserved in plants and plays a key role in RNAi by protecting sRNAs from degradation. Abbreviations: AGO, Argonaute; DCL, Dicer-like protein; RDR, RNA-dependent RNA polymerase.

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appressoria, the specialized hyphal organ formed at the penetration site, indicating differential accumulations of sRNA species during development and infection (53, 98). Furthermore, a 22-nt milRNA, milR236, was shown to target the histone acetyltransferase type B catalytic subunit gene *MoHAT1*, which is required for appressorium formation (82). As such, sRNAs may regulate virulence gene expression in *M. oryzae*.

***Botrytis cinerea*.** *B. cinerea* is a necrotrophic pathogen that causes gray mold diseases in more than 1,400 plant species (127). The *B. cinerea* genome encodes two DCLs, three RDRs, and four AGOs (43). A detailed examination of sRNA profiles in *B. cinerea*–infected *A. thaliana* revealed three 21-nt sRNAs (*Bc*-siR3.1, *Bc*-siR3.2, and *Bc*-siR5) that had increased accumulations in infected tissues. These pathogen-derived siRNAs were found to coprecipitate with AGO1 of *A. thaliana*, indicating that they can potentially regulate host targets. Indeed, *Bc*-siR3.2 and *Bc*-siR5 were predicted to silence defense-related genes in *A. thaliana*, and transgenic plants overexpressing *Bc*-siR3.2 or *Bc*-siR5 showed increased susceptibility to *B. cinerea* (124). Later, another sRNA *Bc*-siR37 was also shown to increase the susceptibility of *A. thaliana* by targeting defense-related genes in transgenic plants (122). These results indicate that *B. cinerea* produces multiple siRNAs that contribute to pathogenesis through cross-species gene silencing. Consistent with this hypothesis, the *Bcdcl1 Bcdcl2* double mutant, but not *Bcdcl1* or *Bcdcl2* single mutants, exhibited reduced virulence activity (124).

Necrotrophic pathogens are generalists with a broad host range. Major virulence factors of necrotrophs are toxins and cell-degrading enzymes, which are usually nonspecific to host species. Studies on the evolutionary dynamics of the siRNA-generating sequences in *B. cinerea* isolates and their target genes in a variety of host plants will provide insight into the conservation of this virulence mechanism. Single mutants of *Bcdcl1* or *Bcdcl2* showed reduced growth and delayed sporulation, and the *Bcdcl1 Bcdcl2* double mutant exhibited a stronger developmental deficiency (124), suggesting that sRNAs also regulate endogenous genes in *B. cinerea*. It will be interesting to

investigate whether the sRNAs that exert gene-silencing functions inside the pathogen can affect pathogenesis.

***Verticillium dahliae*.** *V. dahliae* is a hemibiotrophic pathogen that causes Verticillium wilt, a disease that has devastated many economically important crops (75). The genome of *V. dahliae* encodes three RDRs, two AGOs, a canonical DCL (*Vd*DCL1), and an atypical *Vd*DCL2, which lacks one of the two RNase III domains (66). Mutants of *Vddcl1*, *Vddcl2*, *Vdago1*, and *Vdago2* showed defects in hyphal growth and spore development, indicating a major role of the RNAi pathway in development (66). *V. dahliae* produces sRNAs of 18–25 nt in size without a size preference. A highly expressed 21-nt milRNA, *Vd*milR1, has been functionally characterized. The precursor transcript of *Vd*milR1 is predicted to generate a typical stem-loop structure, similar to miRNAs of plants (66). Unexpectedly, the biogenesis of *Vd*milR1 did not rely on *Vd*DCL1 or *Vd*DCL2; rather, another RNase III domain-containing protein, *Vd*R3, was required for the biogenesis of *Vd*milR1, although the precise activity of this enzyme remains to be determined (117). *Vd*milR1 targets a hypothetical protein-coding gene, *VdHy1*, for TGS associated with increased levels of histone H3K9 methylation. Importantly, a *VdHy1* deletion mutant showed reduced hyphal growth and melanin production when growing in media and markedly reduced virulence in cotton plants, which may or may not be an indirect consequence of the growth defect (66). It is also unknown whether and how *Vd*milR1 may be regulated during infection to derepress *VdHy1* and enhance disease.

***Fusarium graminearum*.** *F. graminearum* causes Fusarium head blight (FHB) of wheat, barley, and other cereals, which not only results in severe yield loss but also mycotoxin contamination of the grain (29). Analysis of the *F. graminearum* genome revealed two DCLs, two AGOs, and five RDRs (28). *Fg*AGO1 and *Fg*DCL2 are required for hairpin RNA-induced gene silencing, confirming the activity of a canonical RNAi pathway in *F. graminearum*. Analysis of sRNAs in mycelia showed a size distribution of 17–32 nt, with the highest number of sequences appearing at 27–28 nt (28); however, a different sRNA profile and size distribution were found in the perithecium, with the highest number of sequences appearing at 24 nt (142). These observations suggest that distinct sRNA populations are produced at different developmental stages. Mutants of *Fgago* and *Fgdcl* genes displayed increased sensitivity to stresses caused by DNA damage or osmotic agents (28) and defects in ascospore formation and discharge (142). However, no detectable changes in mycelial growth or virulence were observed in these mutants (28), indicating that sRNAs may not play a significant role in pathogenesis.

***Puccinia striiformis* f. sp. *tritici*.** *P. striiformis* is an obligate biotrophic fungus that causes stripe rust on wheat (22). Core enzymes of the canonical RNAi pathway include one DCL, one AGO, and two RDRs, but their function in gene silencing has not been comprehensively studied. The sRNA population of *P. striiformis* shows a broad size distribution of 18–40 nt with a pronounced peak at 20–22 nt and another smaller peak at 24 nt. Some predicted sRNA precursors possess a pri-miRNA-like stem-loop structure, indicating a biosynthesis pathway similar to miRNAs in plants and animals; others originate from inverted repeat-containing sequences, similar to siRNAs (120). Whether there are additional dicing enzymes to process these precursors and produce sRNAs with different sizes is unknown. sRNA profiling in infected wheat tissue identified 18 highly accumulated *Pst*-siRNAs (94), including the 20-nt *Pst*-milR1. Produced in a *Pst*DCL-dependent manner, *Pst*-milR1 is predicted to target the *pathogenesis-related 2* (*PR2*) gene of wheat and potentially enhances *P. striiformis* infection. However, infection progression was unchanged when *Pstdcl* was knocked down even though subtle morphological changes were

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observed (120). Further experiments are needed to provide clearer evidence on the function of *Pst*-sRNAs during infection.

In addition to the species discussed above, the sRNA complements of many other phytopathogenic fungi have also been investigated, including *Zymoseptoria tritici* (134), *Puccinia triticina* (39), *Fusarium oxysporum* (26), *Aspergillus flavus* (7), *Metarhizium robertsii* (92), *Curvularia lunata* (86), *Penicillium italicum* (140), and *Valsa mali* (133). These studies relied mainly on sRNA-seq data and bioinformatic predictions of potential targets of the predicted sRNAs. The biogenesis and function of the RNAi pathway and the predicted sRNAs have not been characterized. One exception is *Z. tritici*, a wheat pathogen that causes the Septoria tritici blotch disease. Mutants of *Ztdcl* and *Ztago* genes did not show altered virulence in wheat, suggesting the canonical RNAi pathway is dispensable for pathogenicity. Although some sRNAs of *Z. tritici* were predicted to have potential targets in wheat, silencing of these predicted targets during infection was not observed (72). This study is a good example demonstrating the importance of experimental confirmation of bioinformatic predictions. In particular, the short RNA reads from sequencing data should be confirmed as true sRNAs that are produced through specific biosynthetic pathways and can exert gene-silencing functions. A desirable approach is to determine the sRNA population associated with the AGO complexes, which would lend support to their activity as gene-silencing agents. For organisms in which genetic manipulation is possible, targeted mutagenesis of genes encoding predicted RNAi core enzymes provides unambiguous evidence for the biological significance of sRNAs in the pathogen of interest.

Small RNA from *Phytophthora* Species

Resembling fungi in morphology and lifestyle, the filamentous eukaryotic microorganisms oomycetes belong to the kingdom Stramenopile (12). Some oomycetes, including species of the genus *Phytophthora*, are devastating plant pathogens that cause enormous economic damage in agriculture and forestry (70). Well-studied species include *P. infestans*, which causes the potato and tomato late blight; *P. sojae*, which causes root and stem rot of soybean; and *Phytophthora ramorum*, which causes sudden oak death.

A rich resource in genome sequences of *Phytophthora* and their related oomycetes enables the comprehensive analyses of sRNAs in these organisms. The presence of active RNAi is supported by the well-established target gene knockdown approach, which has been employed as a tool to study gene functions in several *Phytophthora* species by using hairpin or antisense RNA constructs (1, 147). Canonical RNAi core enzymes in *Phytophthora* include a single RDR, two DCLs (14, 44), and variable numbers of AGOs (15). The two DCLs in each *Phytophthora* species form two well-supported clusters, consistent with the accumulation of two major classes of sRNAs, predominantly 21 nt and 25/26 nt, respectively (14, 44, 119). Indeed, production of the 21-nt sRNAs in *P. infestans* has been shown to associate with the activity of *Pi*DCL1 (118); whether the 25/26-nt sRNAs are associated with *Pi*DCL2 is unknown.

Compared to fungal pathogens, *Phytophthora* has an expanded number of AGOs (**Figure 2**), which are also variable in different species. For example, *P. infestans*, *P. ramorum*, and *P. sojae* encode five, six, and nine *AGO* genes, respectively (15). These AGOs form two well-supported clades, with AGO1 from each species clustered together and separated from the other homologs. The expression patterns of *AGO* genes also showed a wide range of diversity. In *P. sojae*, *PsAGO1* is expressed at a high level during all developmental and infection stages. A similar pattern was also observed for *PsDCL*s and *PsRDR*. Other *PsAGO*s had low expression levels overall but some were induced in zoospores, indicating a role in reproduction (15). Co-immunoprecipitation assays in *P. infestans* revealed that *Pi*AGO1 was predominantly associated with 20–22-nt sRNAs and *Pi*AGO4

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primarily bound to 24–26-nt sRNAs, whereas *Pi*AGO5 did not appear to have a size preference for sRNA binding (5). These findings suggest distinct contributions of different AGOs by loading specific sRNA classes for target gene silencing (15). However, knockdown mutants of *Piago1/2* and *Pidcl1* did not show obvious changes in morphology, development, or pathogenicity (119). This could be due to insufficient silencing of the *AGO* and *DCL* genes by the transient RNAi approach used in *P. infestans* or potential functional redundancy of the homologous genes.

The 21-nt sRNAs in *Phytophthora* are primarily derived from inverted repeats and specific gene families, such as Crinkle and Necrosis effectors (CRNs) and type III fibronectins (44), whereas the 25/26-nt sRNAs are predominantly associated with genomic regions with low gene density and harboring transposable elements (44). *Phytophthora* species encode a large complement of cytoplasmic effectors and the effector-encoding genes tend to localize to repeat-rich compartments in the genome (37), raising the possibility of sRNA-mediated regulation. Indeed, abundant sRNAs have been found to be derived from *CRN* genes and LTR retrotransposons in *P. infestans* (44, 118). Furthermore, RNA silencing of transposons may also influence the expression of nearby effector genes through heterochromatin formation (121, 126). As such, sRNAs may affect effector expression, thereby altering the interaction with the host (36). For example, naturally silenced alleles of the effector *Avr3a* were found in field isolates of *P. sojae* that can evade Avr3a-triggered ETI. sRNAs of 24–26 nt accumulated from the *Avr3a* locus, suggesting a link between the silencing of *Avr3a* and sRNA-mediated RNAi (103). In *Phytophthora parasitica*, 25/26-nt sRNAs, but not 21-nt sRNAs, are associated with efficient gene silencing. The 25/26-nt sRNA–generating loci reside predominantly in gene-sparse and repeat-rich regions, which also harbor approximately 50% of the effector genes and some immune elicitor-encoding genes (65). This genome localization bias may allow epigenetic regulation of effector expression, which could facilitate the rapid evolution of *Phytophthora* in the arms race with their hosts (36, 99).

Although *Phytophthora* species have a conserved RNAi pathway, only one conserved sRNA, miR8788, has been identified from *P. infestans*, *P. sojae*, and *P. ramorum*. miR8788 induces cleavage of its target gene, *AAAP*, which encodes an amino acid/auxin permease (44). In addition, many tRNA-derived sRNAs, which regulate gene expression by inducing sequence-specific degradation of target RNAs, were found in *P. sojae* (123). However, the biological significance of target regulation by miR8788 and tRNA-derived sRNAs has not been demonstrated.

SMALL RNA TRAFFICKING BETWEEN HOSTS AND PATHOGENS

sRNAs are mobile molecules that can function beyond the cells from which they are synthesized (85). In plants, both miRNAs and siRNAs have been found to move locally from cell to cell through plasmodesmata and over long distances via the phloem (90). In antiviral immunity, siRNAs may move in advance of the spread of infection to prime silencing of viral RNAs in uninfected cells (41). Whether sRNAs could also prime plant defense against nonviral pathogens by functioning as systemic signaling molecules is an attractive possibility remaining to be tested. Compared to siRNAs, miRNAs are considered to be less mobile and the sites of their synthesis were found to be correlated with the sites of action (4, 100). Nonetheless, local, systemic, and *trans*-species movement of a few plant miRNAs has been reported. Importantly, sRNA mobility is not directly correlated with abundance, indicating a sorting mechanism that could potentially be based on and influenced by biosynthesis, sequence, and structure of the sRNA molecule as well as their interaction with RNA-binding proteins.

Recent research has highlighted *trans*-species gene silencing, in which sRNAs derived from one partner of an interacting pair silence target transcripts in the other organism. Although *trans*-species gene silencing has been indicated in a few plant pathosystems, the current evidence is

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mostly based on sRNA sequencing data obtained from infected plant tissues and bioinformatic prediction of potential targets. In two studies, foreign sRNAs were demonstrated to associate with AGO complexes (40, 124). In these studies, sRNAs produced by the necrotrophic fungal pathogen *B. cinerea* (124) and the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (40) guide target gene silencing in their host plant, *A. thaliana*. Because these host targets are components of the plant immune system, the pathogen sRNAs dampen plant defense and promote disease (**Table 2**). However, how these sRNAs are secreted and translocated from the pathogen into the host cells has not been explored. Therefore, the mechanisms underlying sRNA movement, which is a central process of *trans*-species gene silencing, remain unclear. Here, we focus on discussing the potential mechanisms by which plant sRNAs may traffic to invading pathogens.

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Small RNA Secretion from Plant Cells

In eukaryotes, the general secretory pathways include the classic endoplasmic reticulum (ER)–Golgi route, in which proteins with an N-terminal secretion peptide are translated in membrane-bound polysomes (MBPs) on rough ER and transferred to Golgi apparatus through budding vesicles. Contents in these secretory vesicles are released to the extracellular compartment after being fused to the plasma membrane (104). These contents can be subsequently taken up by another cell through endocytosis. The unconventional Golgi-independent secretion pathway involves the release of EVs whose cargo includes proteins lacking the secretion peptide, RNA, lipids, and metabolic compounds (108). EVs are enclosed in endosomal compartments called multivesicular bodies (MVBs) in the cytosol and released after MVBs are fused with the plasma membrane. The contents of EVs are delivered into the cytosol of a recipient cell after the EV membrane is fused with the plasma membrane of the recipient cell. In animals, RNA cargos are essential for cell–cell communication through EVs (32).

EVs of plants have been investigated for sRNA transportation (**Figure 1**). In *A. thaliana*, secondary siRNAs, including tasiRNAs and *PPR*-siRNAs, that can exert host-induced gene silencing were found to be present in EVs (19, 58). Importantly, mutants of *A. thaliana* genes involved in the exosome formation (a class of EVs) were unable to silence target genes in *B. cinerea*, indicating that exosomes are required for the delivery of tasiRNAs into the fungal cells. Furthermore, the loading of tasiRNAs in the exosomes was selective, suggesting potential sorting mechanisms that facilitate the transport of host-targeting tasiRNAs (19). However, a study on extracellular sRNA profiling found that the overall abundance of the 20–24-nt miRNAs and siRNAs was very low in EVs extracted from the apoplastic space of *A. thaliana*; rather, 10–17-nt tiny RNAs (tyRNAs) were highly enriched in these vesicles (8). Although the function of these tyRNAs in gene silencing is unknown, a recent finding that 14-nt sRNAs in humans can guide sequence-specific cleavage of target transcripts by a specific AGO protein (101) raised the exciting possibility that the EV-cargoed tyRNAs in plants may function as silencing agents. Furthermore, the abundance of *PPR*-siRNAs increased in EVs isolated from *P. capsici*–infected *A. thaliana*, indicating that EV cargos may change after pathogen perception (58). Monitoring the dynamics of EV cargos at various infection stages would help clarify the contribution of EVs to sRNA movement in *trans*-species gene silencing.

Despite the lack of enrichment of 20–24-nt sRNAs in EVs, many secondary siRNAs and some miRNAs were found to be enriched in apoplastic spaces (8), suggesting that these sRNAs could be secreted through an EV-independent route. Intriguingly, the initiation of secondary siRNA production requires the association of the triggering miRNAs with MBPs and rough ER (81). Furthermore, essential proteins for secondary siRNA production, including RDR6, were found in siRNA bodies that are often adjacent to Golgi (141). It is therefore tempting to speculate that the biosynthesis and secretion of secondary siRNAs could be linked, facilitating a potential secretion

of siRNAs via the ER–Golgi route (**Figure 1**). As such, the cytoplasmic partitioning of miRNAs versus siRNAs may underlie the difference in their mobility. It is also important to note that a pool of diverse secondary siRNAs is constitutively produced but can be further induced upon pathogen perception (58), possibly as a surveillance mechanism. Therefore, the export of these siRNAs may be an important mechanism to avoid unintended silencing of endogenous genes.

Small RNA Trafficking at the Host–Pathogen Interaction Interface

Biotrophic/hemibiotrophic filamentous pathogens form haustoria, specialized infection structures at the host interaction interface, to facilitate nutrient uptake and effector delivery. Enveloped by a modified plant plasma membrane called the extrahaustorial membrane (EHM), haustoria are separated from the host cell by the extrahaustorial matrix (EHMx) where the plant cell wall is absent (125). In the haustoriated plant cells, numerous organelles accumulate in the vicinity of the EHM, including Golgi stacks, ER, secretory vesicles, and MVBs, presumably secreting antimicrobial compounds to arrest pathogen colonization (115, 138). It is possible that sRNAs are secreted from plant cells via EV-dependent and/or EV-independent pathways through the EHM and then concentrated in the EHMx for subsequent uptake by the pathogen (**Figure 1**). The lack of plant cell wall in the EHMx may facilitate the secretion of sRNAs. As such, haustoria might be a major gateway for sRNA translocation. Targeted secretion of sRNAs at the host–pathogen interaction interface would also increase the local concentration of sRNAs, thereby enhancing the silencing of target genes in the pathogen.

FUTURE PERSPECTIVES

The past few years have seen rapid development in the research field of sRNAs in plant–pathogen/parasite interactions. sRNA profiles have been documented for many pathogens and plants. Some sRNAs have been functionally characterized for their role in disease development. Accumulating evidence suggests that sRNA-mediated gene silencing may occur in a *trans*-species manner, which is an exciting extension of the known function of sRNAs as mobile regulatory and signaling molecules. Understanding how HIGS works is an important step toward implementing sRNA-based defense in disease management.

Despite these advances, many challenges remain. In the burgeoning area of *trans*-species gene silencing, some major gaps need to be filled. One of the assumptions that is often made without sufficient experimental support is that sRNAs can silence predicted transcripts as long as there is sequence complementarity with a certain level of tolerance for mismatch. It is important to consider that sequence complementarity is not the sole factor dictating silencing efficiency. For example, it has recently been shown that miRNA cleavage efficiency is more affected by target site structure than sequence complementarity (136). This consideration is even more important when it comes to target prediction in another organism. It is important to optimize the target prediction and robustly confirm true targets using genetic and biochemical approaches. Verification of sRNAs loading in the AGO complexes of the combating organism and genetic analysis using mutants of the core enzymes of RNAi would lend key support to gene silencing guided by foreign sRNAs by hijacking the endogenous mechanism.

The efficiency of gene silencing is significantly affected by sRNA stability in addition to RNA structure. In plants, sRNA stability is determined by HEN1-mediated 2′-*O*-methyl modification of the 3′ terminal nucleotide. Nonmethylated sRNAs are unstable and subject to rapid degradation by nucleases (64). Notably, clear orthologs of HEN1 have not been identified from any pathogenic fungi or oomycetes. At least in *P. infestans*, sRNAs were found to have unmodified 3′ terminal nucleotides (118). Therefore, it is interesting to study potential mechanisms utilized by pathogens

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to enhance sRNA stability during cell-to-cell transportation and facilitate efficient loading into plant AGOs. Furthermore, careful investigation is required to discover how pathogen sRNAs can effectively compete with endogenous plant sRNAs to bind to AGO and accumulate to sufficient levels to silence target genes and significantly affect their functions.

Another important research area is sRNA trafficking, in which many questions await answers. EV-independent pathways should be examined for their contribution to sRNA trafficking. Previous studies suggest that miRNA movement can be directional across specific cell–cell interfaces (112). Whether similar mechanisms can support directional trafficking of sRNAs at the host–pathogen interface is worth investigation. For sRNAs that are shuttled by EVs, it is important to elucidate sorting mechanisms that determine the selectivity. In animal parasitic nematodes, AGO-siRNA complexes are loaded in EVs for silencing host gene expression (31). Systemic examination of RNA-binding proteins will provide information on how they may be involved in the sRNA trafficking process. Both mRNA and sRNA are mobile. Thousands of mRNAs have been found to be exchanged between a parasitic plant and its host (74). The current evidence cannot rule out the possibility that RNA precursors may be the mobile molecules that trigger sRNA production in the combating organism. This could potentially remediate the compatibility issue of foreign sRNAs with the AGO complex.

A central concept in host–pathogen interaction is the coevolutionary arms race. An excellent example of this tug-of-war is pathogen effectors and the NLR receptors, both exhibiting patterns of diversifying evolution. If specific sRNAs are used to silence targets in the combating organisms, evolutionary features reflecting the antagonistic interactions are expected to be observed in both sRNA-generating sequences and the target genes. Interestingly, the maize pathogen *U. maydis* has lost the canonical RNAi machinery, possibly due to selective pressure imposed by host sRNA-based defense. Investigation of the evolutionary dynamics of components involved in *trans*-species gene silencing will provide essential insight into the role of sRNAs in host–pathogen interactions. A comparison of these evolutionary dynamics in pathosystems with different infection styles (biotrophy versus necrotrophy; broad host range versus narrow host range) will advance our understanding of this new perspective of host–pathogen interactions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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