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The Cross-kingdom Players: Small RNAs in Focus

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Abstract

Plant pathogenic fungi are the main pathogens that cause crop yield loss and affect global food security. Eukaryotic regulatory small RNAs involved in gene silencing have been found to translocate bidirectionally between hosts and parasites. While Host-Induced Gene Silencing (HIGS) technology has been extensively studied in the genetic function and disease control of many fungal pathogens, the mechanisms of cross-kingdom RNA silencing still needs more exploration. In this review, we summarize the most recent important findings on the cross-kingdom sRNA communication between host plants and fungal pathogens. We discuss not only the interactions of sRNAs between host plants and pathogens that infect them, but also the mechanism of RNAi signal transmission via cross-kingdom sRNAs. Given that HIGS and SIGS (spray-induced gene silencing) technology have been developed and applied in controlling fungal diseases, we compare the advantages of these two technologies and look forward to their applicable prospects.

Keywords: ***Small RNAs; Cross-kingdom RNAi; Crop protection***

Introduction

Small RNAs (sRNAs) are a class of regulatory non-coding RNAs around 20~30 nucleotides (nt) long, many of which are involved in mediating the silencing of gene expression [1]. sRNA-mediated RNA interference (RNAi) is a regulatory mechanism conserved in eukaryotes, where sRNAs play key roles in numerous biological processes, including RNA stability and processing, biotic and abiotic stress response and the regulation of morphological and developmental events [2-4]. sRNAs are generated from hairpin-structured or long double-stranded RNA (dsRNA) by RNase III-like endonucleases named Dicers [5,6].

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Higher plant transcriptomes are enriched in two types of endogenous small RNAs: small interfering RNAs (siRNAs) and microRNAs (miRNAs). siRNAs are a class of 20~24-nt dsRNA molecules that can be derived from the genome or exogenous RNA sequences such as viruses and sense transgene transcripts [2,7]. The silencing effect mediated by these plant cytoplasmic siRNAs is regulated by the RNA-Induced Silencing Complex (RISC) that requires an Argonaute (AGO) protein as the core component to target mRNA transcripts [8,9]. miRNAs are single-stranded non-coding RNAs of typically 20~22-nt in length that are produced from primary miRNAs (pri-miRNAs) containing a stem-loop structure, which are mostly transcribed by RNA polymerase II (Pol II) from regions located between protein-coding genes [1,6]. In plants, Mature miRNAs are incorporated into AGO proteins to direct post-transcriptional gene silencing (PTGS) via transcript cleavage and translation repression. In other cases, they can also trigger the biogenesis of secondary siRNAs [10].

Small RNAs can move from cell to cell through a gating mechanism polarized at defined cell-cell interfaces to modulate plant development, stress responses and immunity [11,12]. Some sRNAs can also move across the boundaries between a host and interacting pathogens or parasites to trigger gene silencing, a mechanism called cross-kingdom RNAi [13]. Plants are susceptible to infection by fungal pathogens with broad host ranges. In fungi, it has been reported that fungal sRNA molecules of *Botrytis cinerea* (*B. cinerea*) are transferred into host plant cells, acting as sRNA effectors to suppress host immunity and achieve infection [14]. This is the first time that fungal pathogen has been found to deliver sRNAs into host cells and hijack plant mRNAs, although it is known for decades that sRNAs may carry long-distance signals in plants [15]. Recently, attention has been focused on mobile sRNAs that mediate cross-kingdom RNAi among host-pathogen interactions. In this review, we summarize the current knowledge of cross-kingdom sRNA silencing, mainly focusing on the plant-fungal pathogen interactions.

Cross-kingdom gene silencing by sRNAs from fungi to host plants

Fungus-derived sRNAs can target plant mRNA transcripts through sequence complementarity. For instance, upon the infection in Arabidopsis, *B. cinerea* Bc-siR3.2 can be loaded into plant AGO1 protein and targets plant mitogen-activated protein kinase 2 (MPK2) and MPK1 transcripts, as well as the tomato MPKKK4, which are involved in plant immunity response to fungal pathogen attack. In addition, an oxidative stress-related gene, peroxiredoxin (PRXIIF) and cell wall-associated kinase (WAK) are targeted by Bc-siR3.1 and Bc-siR5, respectively [14]. Such sRNA effectors are mostly produced by Dicer-like protein 1 (Bc-DCL1) and Bc-DCL2 [16]. Moreover, Bc-siR37 target three immune responsive genes: WRKY7, PMR6, and FEI2, which encode an immune-related transcription factor, a pectinlyase, and a leucine-rich repeat (LRR) receptor kinase, respectively. In a follow-up study, these target genes were suppressed in the transgenic Arabidopsis plants overexpressing Bc-siR37, which exhibited enhanced disease susceptibility to *B. cinerea* [17].

Intriguingly, a novel microRNA-like RNA 1 (milR1) from *Puccinia striiformis* f. sp. tritici (Pst) is exported into wheat and suppresses the wheat pathogenesis-related 2 (PR2) gene to impair wheat resistance to Pst. Silencing of the milR1 precursor resulted in increased wheat resistance to the virulent Pst, while PR2 knockdown plants increased the susceptibility to Pst [18]. Consistent with Bc-siRNAs [14], milR1 may also function as an effector to inhibit the host plant immune defense response [18]. These studies suggest that fungal pathogens utilize a cross-kingdom RNAi strategy to suppress the host innate immune system and ensure the success of infection.

Cross-kingdom gene silencing by sRNAs from host plants to fungi

Like some fungal pathogens can transfer sRNAs into host plants to suppress their immunity, host plants in nature can also export sRNAs into pathogens to induce gene silencing and reduce fungal virulence when they carry suitable RNAi constructs directed against fungal target genes necessary for their pathogenicity. Whether the silencing-inducing RNA molecules are delivered from host plants to pathogens can be tested by assessing fungal development on plants that express RNAi constructs [19]. HIGS technology was first applied by Huang et al. to silence a root-knot nematode parasitism gene by expressing dsRNA in Arabidopsis [20].

Indeed, this powerful technology, termed host-induced gene silencing (HIGS), has long been considered an effective tool to address fungi gene function and control fungal diseases [19,21-23]. Also, it has the potential of accurate multiple disease control by using transgenic plants that express multiple stacked RNAi target sequences, excluding off-targets in the given crop. Recently, the report on a naturally occurring miRNA trafficking between cotton plants and a fungal pathogen has further expanded the understanding and application of HIGS technology [24]. In this study, upon infection with *Verticillium dahliae*, cotton accumulate miR166 and miR159 that target *V. dahliae* genes encoding Ca^{2+} dependent cysteine protease calpain clp-1 (Clp-1) and isotrichodermin C-15 hydroxylase (HiC-15), respectively. These two miRNAs are exported both to fungal hyphae for specific silencing. More importantly, both Clp-1 and HiC-15 transcripts are reduced in the hyphae recovered from *V. dahlia* infected cotton and the fungus mutants with targeted genes knocked out indeed displayed reduced virulence [24].

With the discovery of cross-kingdom sRNAs (**FIG. 1 and TABLE 1**), it becomes intriguing to explore how mobile sRNAs move across the boundary between host plants and fungi. It has been speculated that these sRNA molecules may travel to the fungi via an exosomal pathway [19] since exosomes accumulate at plant-fungus contact sites and vesicles fusion is observed. Additionally, plant multivesicular bodies have been shown to contain small RNAs and other necessary components of the silencing machinery [25]. A recent study in Arabidopsis revealed that plant cells can secrete exosome-like extracellular vesicles (EVs) to deliver sRNAs into fungal pathogen *B. cinerea* [26]. As key mediators of plant-microbe interactions, EVs are essential vehicles of intercellular communication [27]. During the evolutionary arms race with fungal pathogens, Arabidopsis has emerged EVs-mediated cross-kingdom RNAi as a unique method to activate its immune response [26]. However, whether this EV pathway conserves in all forms of life require further elucidation.

HIGS and SIGS

As a highly efficient genetic strategy for controlling sucking insects, nematodes and pathogenic fungi [28], HIGS technology does not require the cultivation of disease-resistant plants. An inverted-repeat transgene with an intron-containing structure can be employed successfully to produce dsRNAs and siRNAs in plants and further specifically shut down the transcripts of parasitic organisms [29] (**FIG. 2**). Hence, it is vital to construct an RNAi-mediated system that can express double-stranded RNA specific to the targeted gene in the transgenic host plant. For example, Xu et al. utilized Tobacco rattle virus (TRV)-based RNAi constructs in cotton plants to silence a regulator of G-protein signaling gene of invaded *V. dahliae* and enhance resistance to this pathogen [22]. Similarly, Song et al. assessed whether three *V. dahliae* virulence genes (Ave1, Sge1, and NLP1) can be used to inhibit Verticillium wilt as silencing targets by transiently expressing TRV: RNAi constructs in tomato.

Subsequently, only HIGS of Sg 1 was not achieved because of a light reduction in Sge1 expression [30]. In all, depending on the suitable target gene chosen, HIGS against pathogens is operational and can be applied to plant protection, especially for the crop plants that are ecologically important and/or of high agronomic values.

The schematic diagram illustrates the transmission of cross-kingdom RNAi signals in plant-fungal pathogen interactions, and how HIGS and SIGS can be used to protect plants against fungal infection. Fungal pathogens deliver sRNA effectors into host plant cells and hijack the host innate immune system (blue arrows and blue block sign). Host plant cells also export HIGS artificial sRNAs or endogenous sRNAs into fungal cells, to silence virulence genes and other important genes for fungi growth (purple arrows and purple block sign). SIGS sRNAs or long dsRNAs, which target fungal pathogenicity-related genes, can be either taken up directly by the eukaryotic pathogen or indirectly move from hosts to pathogen cells (red arrows).

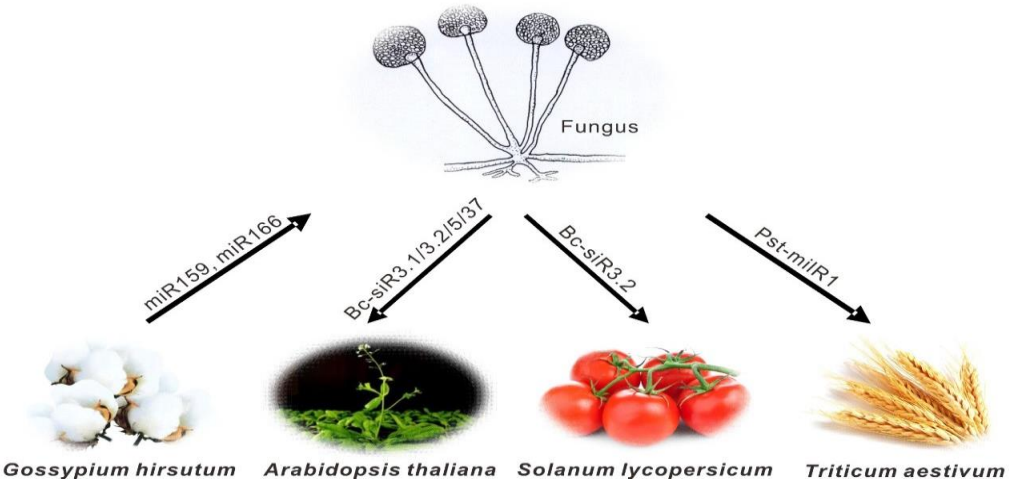

FIG. 1. **Cross-kingdom small regulatory RNAs in plant-pathogen interactions.**

TABLE 1. **Small RNAs and their target genes in plant-fungal pathogen interactions.**

sRNA	From*	To*	Target genes	References
Bc-siR3.2	*B. cinerea*	*A. thaliana*	MPK2 and MPK1	[14]
Bc-siR3.1	*B. cinerea*	*A. thaliana*	PRXIIF	[14]
Bc-siR5	*B. cinerea*	*A. thaliana*	WAK	[14]
Bc-siR3.2	*B. cinerea*	*S. lycopersicum*	MAPKKK4	[14]
Bc-siR37	*B. cinerea*	*A. thaliana*	WRKY7, PMR6 and FEI2	[17]
Pst-milR1	*P. striiformis* f. sp. tritici	*T. aestivum*	PR2	[18]
miR166	*G. hirsutum*	*V. dahliae*	Clp-1	[24]
miR159	*G. hirsutum*	*V. dahliae*	HiC-15	[24]

Note: *Indicate the direction of RNAi transmission signals.

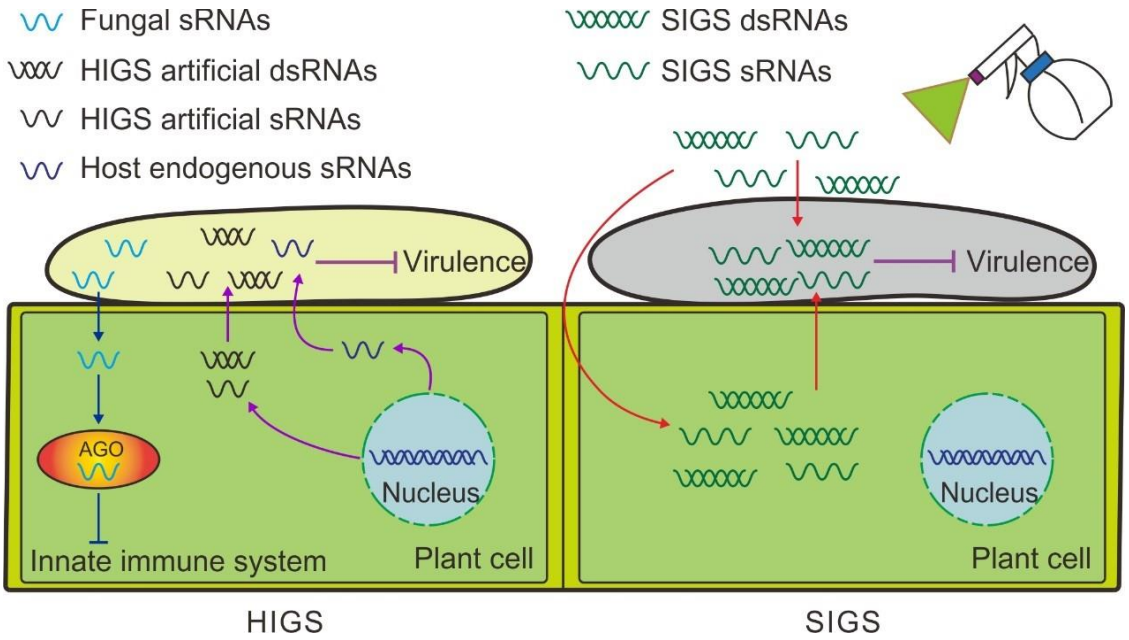

FIG. 2. **Host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS) for controlling fungal pathogens.**

Since *B. cinerea* has been shown to take up external sRNAs and dsRNAs, an alternative technology called spray-induced gene silencing (SIGS), has also been effective for crop protection against pathogens by spraying sRNAs and dsRNAs that target fungal genes on the surface of fruits, vegetables and flowers [16,31] (**FIG. 2**). A recent study demonstrates that spraying barley long dsRNAs that target Fusarium graminearum cytochrome P450 lanosterol C-14α-demethylase (CYP51) gene significantly inhibits the fungal growth. The exogenous long dsRNA is taken up by the plant and transferred in an unmodified form via the vascular system to fungal infection sites where it is processed into siRNAs by fungal DCL1 for its antifungal activity [32]. However, much remains unknown about how exogenous RNAs are taken up by plant and fungal cells, and how these RNAs are transferred from plant cells into fungal cells [33]. Compared with HIGS, the knowledge of SIGS is still limited and more exploration is needed. Overall, there are still obstacles for using SIGS, due to sRNA degradation, cell wall barrier and host plant diversity, etc. which hampers the development of new broad-spectrum environment-friendly fungicides into mass production. Thus, there is still a long way to go from the laboratory to practical applications.

Future Perspectives

Traditional studies of plant-fungi interactions mainly focus on protein modification and/or resistance gene screening; cross-kingdom sRNA has opened up a new field for future research. With the rapid development of deep sRNA sequencing technology and comparative genomics, more cross-kingdom sRNAs will be discovered, along with the pathogen effectors. It is expected to reveal the detailed mechanism of bidirectional RNAi signal transmission, to facilitate a full understanding of sRNAs in nature.

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