CROSS-KINGDOM RNA INTERFERENCE: A NOVEL STRATEGY FOR PLANT DISEASE MANAGEMENT

Abstract

Small RNAs (sRNAs) are short, non-coding RNA molecules that play a crucial role in regulating gene expression. They include microRNAs (miRNAs) and small interfering RNAs (siRNAs) and are involved in controlling plant immunity against pathogens and pests. Recent research has revealed that sRNAs can move between different species and silence genes, a process called cross-kingdom RNA interference (RNAi). This discovery has led to the development of host-induced gene silencing (HIGS), where transgenic plants are engineered to produce double-stranded RNA (dsRNA) that targets specific pathogen genes. This RNA-based approach offers a promising alternative to agrochemicals and transgenic crops with potential health and environmental risks. By applying dsRNAs or sRNAs directly onto host plants or post-harvest products, a technique known as spray-induced gene silencing (SIGS), effective disease management can be achieved without the need for transgenic methods. This chapter explores recent advancements in using cross-kingdom RNAi to manage plant diseases.

Keywords: Cross-kingdom RNA interference, small interfering RNA, microRNAs (miRNAs), host-induced gene silencing, spray-induced gene silencing, disease management.

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I. INTRODUCTION

Small RNAs, including small interfering RNAs (siRNAs) and microRNAs (miRNAs), are a class of non-coding RNAs that typically range from 20 to 30 nucleotides in length. They play important roles in regulating gene expression [1]. SiRNAs, which are about 20–24 nucleotides long, are double-stranded RNA molecules produced from longer precursor molecules derived from the organism's genome or external sources like viruses and transgenic transcripts [2–3]. Conversely, miRNAs are single-stranded non-coding RNAs that are approximately 20 to 22 nucleotides long. They are derived from primary miRNAs (pri-miRNAs) which are mainly transcribed from regions located between protein-coding genes. These pri-miRNAs have a distinct stem-loop structure [4–5].

The regulation of plant immunity against pests and pathogens (such as fungi, bacteria, oomycetes, and viruses) relies significantly on sRNA-mediated RNA interference (RNAi). Additionally, sRNAs originating from pests and diseases play a crucial role in monitoring their virulence. While it was previously understood that sRNAs could move within cells and tissues of a single organism, recent evidence supports the idea that some of these sRNAs can transfer across different species, leading to the silencing of genes in the recipient organism. This phenomenon is known as 'cross-kingdom RNAi.' By targeting the genes responsible for pathogenicity in eukaryotic pathogens and pests, sRNAs produced within the plant system can effectively suppress them. This discovery has paved the way for the development of RNA-based pesticide formulations that are highly specific and adaptable for controlling multiple diseases simultaneously using cross-kingdom RNAi [6].

Certain pathogens possess the ability to uptake RNAs from the environment, a process known as 'environmental RNAi' [7]. Leveraging this knowledge, host-induced gene silencing (HIGS) technology has emerged, enabling the evolution of a recent approach to plant protection called transgene-mediated cross-kingdom RNAi or 'spray-induced gene silencing' (SIGS). In SIGS, dsRNAs and sRNAs targeting the genes of pathogens are sprayed onto plant surfaces to suppress their virulence [6]. This chapter comprehensively summarizes our current understanding and the practical applications of cross-kingdom RNAi.

To sum up, the current understanding of cross-kingdom RNAi has revealed the potential of sRNAs in regulating gene expression across different organisms. This knowledge has led to the development of innovative RNA-based pesticide formulations and approaches like SIGS, which offer effective means of controlling pests and diseases in the realm of agriculture.

II. THE CROSS-KINGDOM RNA INTERFERENCE

RNAi is a universally observed biological mechanism that controls the expression of protein-coding genes in response to the presence of double-stranded RNA (dsRNA). Evidence suggests that RNAi can be both pathogen as well as host induced. Naturally, in most of the plant-microbe interactions,cross-kingdom RNAi occurs[8]. Pathogenic small RNAs play a role in cross-kingdom RNAi within plant hosts, serving as a unique type of pathogen effector that hinder host immunity to facilitate successful infection [9-10].Cross-kingdom transfer of small RNAs (sRNA) and the subsequent silencing phenomenon are observed in various plant-patho systems, including interactions with fungal pathogens such as

Botrytis cinerea, Verticillium dahliae, Blumeriagraminis, Sclerotinia sclerotiorum, and *Phytophthora infestans.*

Botrytis cinerea, an important fungal pathogen causing pre-and post-harvest diseases in many crops delivers sRNAs(Bc–sRNAs)that bind to the host argonaute-RISC component 1 (AGO1) gene and hijack the host RNAi pathwaysand suppresses the immunity system [8,11].Furthermore, the sRNA Bc-siR37 derived from *Botrytis cinerea* has the ability to suppress the plant's defense mechanisms by reducing the expression of several target genes within the host[12]. *Sclerotiniasclerotiorum* produces at least 374 distinct sRNAs during infection which target the functional domains associated with immunity of *Arabidopsis thaliana*[13].Zhang et al. [14] showed that when cotton plants are infected by *Verticillium dahliae*, they enhance the production of microRNA166 (miR166) and miR159, which are then transported to the fungal hyphae to facilitate targeted silencing.The expression of two critical genes in *Verticillium dahliae*, namely Clp-1 (encoding a Ca2+-dependent cysteine protease) and HiC-15 (encoding an isotrichodermin C-15 hydroxylase), which are essential for the pathogenicity of the fungus, is specifically inhibited by miR166 and miR159, respectively. In experimental conditions, the silencing of these pathogenic genes by small RNAs produced by the host plant has been demonstrated to limit fungal growth, as observed in powdery mildew studies [15]. On the other hand, the oomycete pathogen *Phytophthora infestans* employs RxLR-type effectors that possess RNA-silencing suppressor capabilities [16-17]. Hence, the cross-kingdom transfer and silencing of RNA appear to play a crucial role in the pathogenicity of various fungal and filamentous pathogens, as well as in plant resistance mechanisms.

III. FORMATION OF SMALL RNAS IN PLANTS

In the plant system, miRNAs are produced from hairpin-shaped precursors that contain 64 to 303 nucleotides, whereas in animals, miRNA precursors are usually 60 to 70 nucleotides in size [18-19]. These miRNAs are generated from specific genes called MIR genes. The first step involves the synthesis of primary miRNAs (pri-miRNAs) by an enzyme called RNA polymerase II (RNA pol II) within the nucleus. The processing of pri-miRNAs into mature miRNA duplexes occurs through the action of Dicer-like 1 (DCL1), a ribonuclease III-like enzyme, along with the involvement of Hyponastic Leaves 1 (HYL1) protein. The resulting miRNA duplex consists of two strands: miRNA and miRNA*. The miRNA* strand possesses a 2-nucleotide overhang at its 3' end [20]. The miRNA duplex is methylated by a methyl-esterase known as Hua Enhancer-1 (HEN1), which is present in both plants and animals [21]. Methylation plays a crucial role in protecting RNA from degradation by exonucleases and from uridylation, which involves the addition of a short poly-U tail to unmethylated miRNAs. This uridylation decreases the stability of miRNAs, making them more prone to decay. Once the miRNA duplex matures, it is transported to the cytoplasm with the assistance of a nuclear membrane protein known as HASTY (HST) [22-25]. Following that, an unidentified helicase protein unwinds the miRNA-miRNA* duplex, exposing the mature miRNA to the RNA-induced silencing complex (RISC). The mature miRNA, bound to RISC containing the AGO protein, then either cleaves or represses the translation of specific target messenger RNAs (mRNAs) by guiding the RISC to the target mRNA [26-27].

SiRNAs and miRNAs have a close connection but differ in their origin, structure, associated effector proteins, and mechanism of action [4, 28]. There are two types of siRNAs: trans-acting siRNAs (ta-siRNAs) and repeat-associated siRNAs (ra-siRNAs), which vary in their precursor molecules and synthesis steps. Ta-siRNAs originate from non-coding RNA (ncRNA) precursors, whereas Ra-siRNAs are produced from transposable elements and repetitive sequences.The synthesis of ssRNA precursor by miRNA-dependent processing represents the most distinctive aspect of Ta-siRNA biogenesis [29-31] on the other hand, ssRNA for Ra-siRNAs is synthesized by DNA-dependent RNA polymerase IV from the heterochromatic locus.Later, RNA-dependent RNA polymerases (RDRPs) duplicate the ssRNA precursors in both siRNAs to produce dsRNA precursors. The rest of the process of siRNA synthesis is the same as that of miRNA, with the exception of the types of enzymes and proteins involved [32].

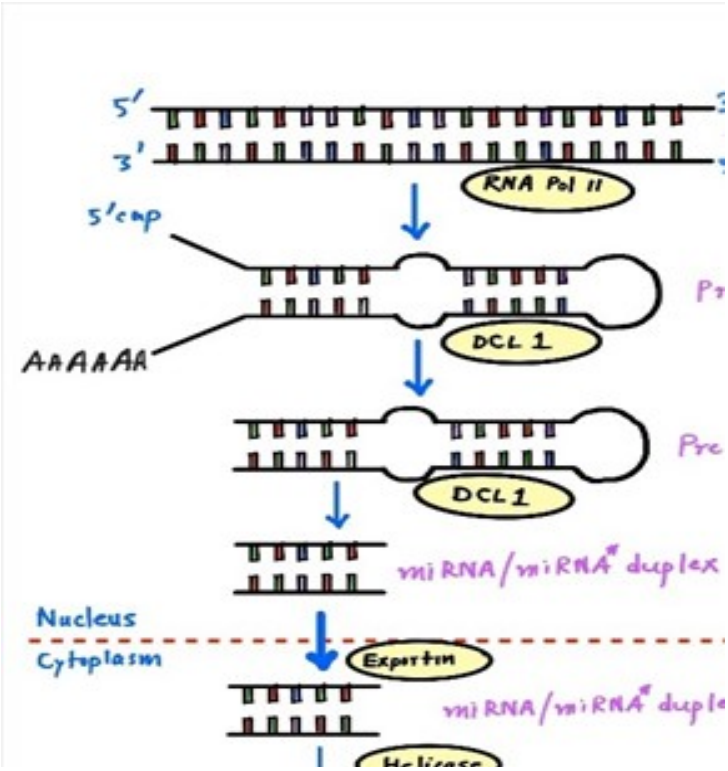

Figure 1: Mechanism of miRNA synthesis in plants[33].

IV. CROSS-KINGDOM MOVEMENT OF sRNAs

The movement of sRNAs within organisms or between different kingdoms is crucial for regulating the expression of specific target genes. This movement allows the transmission of RNA-silencing signals from one cell type to another, even across long distances, thereby influencing gene expression in distant cells and tissues. This phenomenon is referred to as "systemic acquired silencing" (SAS). It has been extensively studied and documented [34-36]. The sRNA population involved in this mobile transfer often exhibits differences compared to the total sRNA population within cells, indicating that there is a selective process taking place during the transfer to ensure the inclusion of potentially functional sRNAs [37-39]. This selection process may involve various factors such as RNA-binding

proteins (RBPs), AGOs, packaging into the extracellular vesicles (EVs), or othermechanisms of transport [9]. Recently, a gating mechanism has been discovered at specific cell-cell interfaces that precisely regulate the transport of miRNAs, preventing their long-distance movement from shoot to root [40].

The movement of sRNAs within an organism, specifically between cells, is of utmost importance in governing gene expression within the organism, thereby playing a pivotal role in its regulatory mechanisms. However, the cross-kingdom mobility of these RNAs is of significant importance to both hosts and pathogens, as it influences resistance mechanisms in the host and pathogenicity in the pathogens [33].

1. **Transfer of sRNAs from Pathogens to Host Plants:** Crop yield losses and their impact on global food security are primarily caused by plant pathogenic fungi. Plants are vulnerable to a wide range of fungal pathogens. In nature, resistance to these pathogens is the norm, while disease development is the exception. For a pathogen to successfully cause disease, it must possess sophisticated mechanisms to overcome the defense barriers of the host [41].

   Pathogens have evolved mechanisms to manipulate host gene expression and evade defense mechanisms. One such mechanism is called pathogen-modulated RNA interference, where small RNA molecules originating from pathogens can act as effectors to alter host gene expression. These sRNAs can undermine the host's defense strategies by targeting specific genes. For example, a study by Wang et al. [42] found that a novel microRNA (miRNA) called Pst-milR1 in *Puccinia striiformis* f. sp. *tritici* targets the pathogenesis-related 2 (PR2) gene in wheat, potentially suppressing the host's defense strategy. Similarly, sRNAs derived from *Botrytis cinerea*, known as Bc-sRNAs, bind to Argonaute 1 (AGO1) and hijack the host RNAi machinery, leading to the selective silencing of host immunity genes [11]. This indicates that *B. cinerea* transfers virulent sRNA molecules into host plant cells to suppress host immunity as a counter-defense strategy for successful infection. Furthermore, bidirectional interactions between pathogens and hosts have been identified. For instance, sRNAs produced by *Plasmoparaviticola* trigger the cleavage of grapevine (*Vitis vinifera*) genes, while grapevine-derived sRNAs target *P. viticola* mRNAs [43]. These examples highlight the intricate interplay between pathogens and their host plants, where sRNAs play a crucial role in modulating gene expression to facilitate infection or counter host defense mechanisms.

2. **Transfer of sRNAs From Host Plants to Pathogens**: Transfer of miRNAs between different kingdoms has been observed during interactions between hosts and pathogens, resulting in the inhibition of the pathogen's invasive capabilities [44]. Plants encounter attacks from various pathogens, such as bacteria, fungi, mycoplasma, nematodes, viruses, viroids, and parasites, and have developed defense strategies to counteract them [45-46]. In a sophisticated manner, plants employ miRNA-mediated transcriptional or post-transcriptional silencing of pathogenic mRNA, specifically targeting virulence genes. This mechanism forms a part of the plant's resistance strategy against pathogens.

   The host organisms possess RNA interference (RNAi) silencing machinery that can directly target the RNA genome and related transcripts of various pathogens like

viruses, virus satellites, and viroids to regulate their accumulation [47]. This silencing process involves the transportation of specific small RNAs (sRNAs) from plants, including miRNAs, which induce gene silencing in pathogenic fungi, thereby enhancing disease resistance [48-49]. For example, in*Arabidopsis*, siRNAs can enter *Phytophthora* through extracellular vesicles during infection, contributing to the development of resistance [48]. Additionally, *Arabidopsis* miR166 has been observed to be exported to *V. dahliae* fungal hyphae, resulting in the suppression of pathogenicity [50]. In cotton plants infected with *V.dahliae*, the accumulation of miR166 and miR159 has been observed, which targets *V. dahliae* genes encoding a Ca^{2+}dependent cysteine protease (Clp-1) and an isotrichodermin C-15 hydroxylase (HiC-15), respectively [14]. Wheat plants restrict disease development by targeting specific genes of *Puccinia striiformis* f. sp. *tritici*, such as calcineurin homologs Pscna1/Pscnb1 and MAPK kinase gene PsFU27, resulting in reduced hyphal extension, decreased uredospore production, and induction of necrosis in plant cells [51-52]. Targeting the PsCK1 gene (a PKA catalytic subunit gene) of *P. striiformis* f. sp. *tritici* also leads to a reduction in disease progression, significantly reducing the length of infection hyphae and disease symptoms [52]. Various other interactions involving RNA and siRNA between plants and fungi have been summarized in Table 1, resulting in either resistance or disease outcomes.

Table 1: Cross-Kingdom RNA Interaction between Plants and Fungi

From	To	Target Genes	Involved sRNA	Reference
B. cinerea	*S. lycopersicum*	*MAPKKK4*	Bc-siR3.2	[11]
B. cinerea	*A. thaliana*	*PRXIIF*	Bc-siR3.1	[11]
B. cinerea	*A. thaliana*	*WAK*	Bc-siR5	[11]
B. cinerea	*A. thaliana*	*MPK2 and MPK1*	Bc-siR3.2	[11]
G. hirsutum	*V. dahliae*	*Clp-1*	miR166	[14]
G. hirsutum	*V. dahliae*	*Clp-1*	miR166	[14]
P. *striiformis* f.sp. *tritici*	*T. aestivum*	*PR2*	Pst-milR1	[42]
B. cinerea	*A. thaliana*	*WRKY7, PMR6 &FEI2*	Bc-siR37	[42]
GRSPaV	*V. vinifera*	*VPS55*	vsiR6978	[53]
GFkV	*V. vinifera*	*S2P metalloprotease*	vsiR1378	[53]

V. POTENTIAL OF CROSS-KINGDOM RNAI APPLICATION IN PLANT DISEASE MANAGEMENT

The transfer of miRNAs between different kingdoms presents promising opportunities for the development of eco-friendly crop protection strategies. For instance, the transfer of miRNA159 and miRNA166 from cotton to the pathogenic fungus *Verticillium dahliae* has been demonstrated to provide resistance [15]. As mentioned earlier, numerous studies have highlighted the horizontal transfer of miRNAs among plants, animals, and microbes. This

transfer facilitates the regulation of gene expression in both host and pathogenic organisms, thereby contributing to the protection of crops in agricultural production. The primary objective of miRNA transfer from pathogens to hosts is to suppress plant defense mechanisms, acting as a counter-defense strategy employed by the pathogens. Wang et al. [12] demonstrated bidirectional RNAi, indicating that the cross-kingdom transfer of miRNAs can suppress the virulence of plant pathogens and protect crops. Another study by Wang et al. [12] revealed that fungal pathogens can uptake sRNAs from the environment, leading to a loss of virulence. For instance, the fungal pathogen *Botrytis cinerea*, responsible for grey mold disease, has been targeted using externally applied sRNAs and dsRNA through spraying on the surface of fruits, vegetables, and flowers, resulting in the suppression of fungal pathogenic genes and protection against plant infections. This discovery opens up new possibilities for managing plant diseases through spray-induced gene silencing (SIGS) [6].

1. **Spray-induced Gene Silencing (SIGS) in Plant Disease Management:** Crop production currently relies heavily on chemical fungicides, which can leave pesticide residues and contribute to the development of resistance in pathogens. Therefore, there is a growing need for an alternative and environmentally friendly method to protect crops. The third green revolution in modern agriculture is on the horizon, and understanding reverse genetics in gene functional characterization holds significant potential for managing agricultural pests. The RNA interference (RNAi) technology offers a practical approach to plant protection, with spray-induced gene silencing (SIGS) being the most effective method for crop protection [54]. SIGS has been observed to successfully silence target genes in viruses [55] and fungi [6, 56-57]. Notably, *Fusarium graminearum* and *Botrytis cinerea* have been effectively controlled using SIGS, which mimics host-induced gene silencing (HIGS) without requiring the modification of plant genomes. The sprayed RNAs can enter fungal cells through two possible pathways: either through plant cells and transferred to the pathogenic fungi, or directly taken up by fungal cells. These RNAs then exert their effects in two ways: by activating the plant's RNAi machinery in plant cells or by directly activating the fungal RNAi machinery in fungal cells. Koch et al. [56] demonstrated successful suppression of the CYP51 gene in barley, providing resistance against *F. graminearum* through SIGS. They also demonstrated the effectiveness of silencing green fluorescent protein (GFP) expression in a GFP-expressing strain of *Fusarium graminearum* by spraying RNA fragments derived from jellyfish GFP onto barley leaves. This finding indicates that small RNA-induced gene silencing (SIGS) is not restricted to specific sequences and has the potential to target essential genes in different interacting pathogens. Additionally, McLoughlin et al. [57] found that spraying dsRNA can inhibit the growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum* on *Brassica napus*.

   SIGS offers a technologically simple and socially accepted approach to crop protection, as it does not involve the generation of genetically modified organisms (GMOs). It is considered safe for consumption and allows effective control of fungi even after harvesting [8, 58]. SIGS has the advantage of simultaneously targeting multiple pathogens, making it practical for managing pests and diseases that affect various crops [59]. Another benefit is that RNA degrades relatively quickly, so any potential accumulation in the environment is not expected to be hazardous, considering that nucleic acids are naturally present [60]. Therefore, RNAi-based pesticides offer an

environmentally friendly alternative to conventional agrochemical products and genetically engineered plant solutions [12].

Table 2: SIGS in plant-pathogen interactions.

Host Plant	Target Pathogens	Target Genes	Concentration of dsRNA	Reference
T. aestivum	*F. asiaticum*	*Myosin 5*	0.1 pM	[61]
A. thaliana	*V. dahliae*	*DCL*	20 ng/µL	[8]
A. thaliana, etc.	*B. cinerea*	*DCL1/2*	20 ng/µL	[8]
H. vulgare	*F. graminearum*	*CYP51*	20 ng/µL	[56]
B. napus	*B. cinerea*	BC1G_04955, etc.	42 ng/µL	[57]
B. napus	*S. sclerotiorum*	SS1G_01703, etc.	20 ng/µL	[57]
A. thaliana	*S. sclerotiorum*	SS1G_03208, etc.	20 ng/µL	[57]

VI. CONSTRAINTS

RNAi has emerged as a promising approach to develop bio-fungicides, providing a sustainable and eco-friendly alternative to conventional chemical fungicides for combating pests and fungal pathogens. Nevertheless, successful large-scale production and commercialization of RNAi-based bio-fungicides require addressing challenges, with a significant hurdle being the uptake efficiency of these bio-fungicides by target pathogens. A study conducted by Qiao et al. [62] demonstrated that not all pathogens possess the ability to efficiently capture long and/or small dsRNAs crucial for effective RNAi-based control. This variation in uptake efficiency significantly impacts the bio-fungicides' success in managing specific pathogens, underscoring the need to identify factors influencing dsRNA uptake. Comprehending intricate mechanisms involved in the uptake of external RNAs by both plant and fungal cells is imperative to unlock the full potential of RNAi-based bio-fungicides. This understanding is essential for designing targeted delivery systems that efficiently and selectively deliver dsRNAs to pathogens. Equally crucial is understanding RNA transport processes between plant and fungal cells, as this influences the overall efficacy and specificity of the bio-fungicide.

Overcoming challenges in RNAi-based bio-fungicides can also be achieved through improved application strategies. One promising approach involves stabilizing RNAs by incorporating chemical reagents during application, enhancing their stability and protection from degradation. This can increase the overall strength and longevity of plant protection against pathogens.Despite challenges, the potential benefits of RNAi-based fungicides are significant. They offer a sustainable solution for managing crop diseases while reducing the environmental impact associated with conventional chemical fungicides. By specifically targeting harmful pathogens and sparing beneficial organisms, RNAi-based bio-fungicides

contribute to improved crop yields and food safety.To seize these opportunities and overcome current limitations, continuous research and collaboration between academia, industry, and regulatory bodies are vital. Further investigations into uptake mechanisms, RNA transport, and innovative delivery systems are necessary to optimize RNAi-based bio-fungicide performance. Rigorous field trials and assessments are equally essential to evaluate safety, efficacy, and economic viability under diverse environmental conditions.

VII. SUMMARY

Cross-kingdom RNA interference (RNAi) is a process in which small RNA molecules regulate gene expression between different organisms. In the context of plants and pathogens, small RNAs known as microRNAs (miRNAs) and small interfering RNAs (siRNAs) play a significant role. Pathogens can produce sRNAs to interfere with host immunity and facilitate infection, while host plants can produce sRNAs to target pathogen genes and suppress their virulence. The biogenesis of sRNAs in plants involves processing primary miRNAs (pri-miRNAs) into mature miRNAs and siRNAs, which then bind to the RNA-induced silencing complex (RISC) to degrade or repress target messenger RNAs (mRNAs).The movement of sRNAs within organisms or between kingdoms is crucial for gene regulation, allowing the RNAi signal to propagate over long distances. Factors such as argonaute proteins, RNA-binding proteins, and packaging into extracellular vesicles influence the selection and transport of sRNAs during their movement. Cross-kingdom RNAi impacts host-pathogen interactions, where sRNAs can be transferred between pathogens and host plants to modulate gene expression, suppress immunity, and inhibit pathogen virulence. Understanding the mechanisms and dynamics of cross-kingdom RNAi has important implications for plant protection. It offers potential applications in developing RNA-based pesticides to control diseases and pests in crops. Harnessing the power of RNA-based approaches can provide new strategies for managing plant diseases and improving crop yield.

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