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Small RNAs—The Secret Agents in the Plant-Pathogen Interactions

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Abstract

Eukaryotic regulatory small RNAs (sRNAs) that induce RNA interference (RNAi) are involved in a plethora of biological processes, including host immunity and pathogen virulence. In plants, diverse classes of sRNAs contribute to the regulation of host innate immunity. These immune-regulatory sRNAs operate through distinct RNAi pathways that trigger transcriptional or post-transcriptional gene silencing. Similarly, many pathogen-derived sRNAs also regulate pathogen virulence. Remarkably, the influence of regulatory sRNAs is not limited to the individual organism in which they are generated. It can sometimes extend to interacting species from even different kingdoms. There they trigger gene silencing in the interacting organism, a phenomenon called cross-kingdom RNAi. This is exhibited in advanced pathogens and parasites that produce sRNAs to suppress host immunity. Conversely, in host-induced gene silencing (HIGS), diverse plants are engineered to trigger RNAi against pathogens and pests to confer host resistance. Cross-kingdom RNAi opens up a vastly unexplored area of research on mobile sRNAs in the battlefield between hosts and pathogens.

Introduction

Eukaryotic non-coding small RNAs (sRNAs) are generated by endoribonucleases DICER or DICER-like (DCL) and are loaded into Argonaute (AGO) proteins to induce silencing of genes with complementary sequences. This mechanism is referred to as RNA interference (RNAi). In plants, sRNAs are divided into two subgroups, small interfering RNAs (siRNAs) and microRNAs (miRNAs), based on their precursor structures and biogenesis pathways. Both miRNAs and siRNAs play a pivotal role in regulating and fine-tuning gene expression in diverse cellular processes such as development and growth, genome integrity, epigenetic inheritance, and cellular stress responses, including host immunity [1-4]. Similarly, sRNAs from eukaryotic plant pathogens, pests, and symbionts also play an important regulatory role in developmental processes and pathogenicity [3,5,6]. Remarkably, some sRNAs are mobile signals in plants that transmit gene silencing from cell to cell, or systemically over a long

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distance [7-10]. Recent attention has been focused on mobile sRNAs that mediate cross-kingdom RNAi in host-pathogen interactions [3,11,12].

Cross-kingdom RNAi is the phenomenon in which gene silencing is induced between unrelated species from different kingdoms, such as a plant host and its interacting microorganism or pest. It requires the translocation of a gene-silencing trigger from a donor into an interacting recipient. Indeed, interaction with other organisms by way of cross-kingdom RNAi has been observed in plant and animal systems [3,11,12]. Cross-kingdom RNAi can occur from the host to the pest/pathogen/parasite/symbiont or *vice versa*. The most prominent example of cross-kingdom RNAi from a plant to its interacting microorganism is host-induced gene silencing (HIGS), a phenomenon in which a plant-produced RNAi signal triggers silencing of a pathogen gene [13,14]. Conversely, sRNAs produced by pathogens and parasites can also translocate into host cells and trigger gene silencing of host genes [5,11,12,15,16]. Advanced pathogens hijack the host RNAi pathways and suppress host immunity genes to facilitate infection [3,12]. This review focuses on the roles of plant- and pathogen-derived sRNAs in host immunity, and pathogen virulence, respectively.

Plant endogenous sRNAs and sRNA pathway components regulate host innate immunity

In plants, diverse classes of endogenous sRNAs, including miRNAs and siRNAs, are involved in regulating and fine-tuning defense responses against pathogens and pests [3,17-20]. miRNAs and siRNAs are involved in the activation of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [21-24] and effector-triggered immunity (ETI) [25-30]. Normally, these sRNAs are either induced or down-regulated upon pathogen attack in order to suppress expression or to release suppression of their targets [20].

Many proteins of sRNA pathways are involved in immune response by manipulating sRNA biogenesis and function, such as DCLs that generate sRNAs, AGOs that execute sRNA-directed target gene suppression, and RNA-dependent RNA polymerase (RDRs) that produce double-stranded sRNA precursors. The *Arabidopsis* genome encodes 4 DCL proteins. DCL1 is the key protein in miRNA production, and several miRNAs it produces are associated with PTI and ETI against bacterial and fungal pathogens. Consistent with this observation, the *Arabidopsis* mutants *dcl1-9* and *dcl1-7* showed enhanced susceptibility toward bacterial [31] and fungal [16] infection. These findings emphasize the notion that miRNAs participate in regulation of immune response. DCL4 is mainly involved in siRNA production and is important in antiviral, antibacterial, and antifungal defense [25,32].

There are 10 AGO proteins in *Arabidopsis* [33]. Only AGO2 is highly induced by bacterial infection [24], and the *ago2-1* mutant is more susceptible to both virulent and avirulent strains of *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) [24]. Deep sequencing analysis of AGO2-associated sRNAs after immunoprecipitation identified several miRNA*s. miRNA393* is one of the most abundant sRNAs loaded into AGO2, resulting in the suppression of MEMB12 upon infection of *Pst* (AvrRpt2), and promoting secretion of pathogenesis-related (PR) proteins. Interestingly, the complementary strand of miR393*, miR393, functions through AGO1 to induce antibacterial immune response [34]. This study

has demonstrated that miRNA*s, formerly considered non-functional byproducts of miRNAs, can be functional in inducing gene silencing [35]. Similar phenomena have also been observed in animal systems [36,37]. AGO1 generally plays a positive role in plant immunity. The *ago1-25* and *ago1-27* mutants are hindered in PAMP-perception and in antibacterial immunity [22]. However, *ago1* mutants showed enhanced disease resistance against certain fungal pathogens [16,38], indicating a sophisticated role of plant AGO1 protein in plant-fungal interactions, which is discussed in greater detail below. The *Arabidopsis* genome encodes six RDRs, of which RDR6 is involved in secondary siRNA production. The *rdr6* mutant exhibits enhanced susceptibility to fungal pathogens [38] and an avirulent bacterial *Pst* strain carrying the AvrRpt2 effector gene [26], while the *rdr6* mutant exhibits enhanced basal resistance toward a virulent strain of *Pst* [39,40]. Moreover, mutation in a RDR6 interacting protein SGS3 also enhances susceptibility to *Verticillium dahliae* [38], suggesting that the sRNA pathway is generally required for antifungal resistance in plants.

Furthermore, heterochromatic siRNAs (hcsiRNAs) direct DNA methylation and/or histone modifications to induce silencing of transposons, repeats and genes at the transcriptional level. This hcsiRNA-mediated so-called RNA-directed DNA methylation (RdDM) pathway also regulates immune responses [41,42]. RdDM mutants display altered disease phenotypes to bacteria or fungal pathogen infection. For instance, the triple mutant of the non-CG loci methyltransferases, *drm1-2/drm2-2/cmt3-11* (*ddc*), the mutant in which the largest subunit of Pol IV being mutated *nrpd1a*, a chromatin remodeling protein mutant *drd-1*, and the *dcl2/dcl3/dcl4* triple mutant, all show enhanced resistance to *Pst* [41]. In addition, expression of many of the RdDM pathway genes are down-regulated upon treatment with the bacterial PAMP trigger flg22, supporting the notion that RdDM transcriptionally controls the expression of antibacterial defense genes [42]. Consistent with this, most of these RdDM pathway genes are also repressed during bacterial infection, which leads to demethylation and activation of several defense genes [42]. Furthermore, *ROS1*, which encodes for a 5-methylcytosine DNA glycosylase that initiates active DNA demethylation, is repressed upon flg22 treatment, and *ros1* mutant exhibits enhanced susceptibility to *Pst* [42], suggesting that active DNA demethylation is part of the regulatory circuit for gene activation in response to pathogen attacks. The *ros1/dml2/dml3 (rdd)* triple mutant, and the RdDM pathway mutants *ago4* and *nrpe1* are more susceptible to *Fusarium oxysporum* [43], and microarray analysis indicates that a much larger group of genes was differentially expressed in the RdDM mutants *nrpd1* and *nrpe1* than in the *rdd* mutant. Obviously, RdDM or DNA demethylation re-arranges the transcriptional status of immunity genes. The *ago4*, *drd1*, *rdr2*, *drm1 drm2*, and *nrpd2* mutants showed enhanced susceptibility toward necrotrophic fungal pathogens *Botrytis cinerea* and *Plectospherella cucumerina*, which is contrary to the increased resistance against bacterial pathogens [44]. Chromatin immunoprecipitation revealed that the RdDM pathway epigenetically controls salicylic acid–dependent defense responsive genes, which are activated in *nrpd2* and other RdDM mutants [41,42], leading to enhanced resistance to bacterial pathogens. However, these mutants compromise jasmonic acid-dependent defense responses and enhance susceptibility to necrotrophs. Furthermore, trans-generational systemic acquired resistance was observed in *Arabidopsis*, which was dependent on RdDM-mediated hypomethylation at non-CG sites [45].

Thus, plant endogenous sRNAs and sRNA pathway components are critical factors in regulating and fine-tuning host immune responses (Figure 1A) against an array of pathogens and pests, including bacteria, fungi, oomycetes, nematodes, and insect pests.

Microbial sRNAs regulate pathogen virulence

Although plant sRNAs involved in host defense have been extensively studied, relatively little is known about the function of pathogen-derived sRNAs. Recent studies have demonstrated that microbial sRNAs play a regulatory role in pathogen virulence. Both prokaryotic and eukaryotic organisms produce a plethora of non-coding sRNAs; however, the structure of bacterial non-coding sRNAs differs significantly from that of eukaryotic sRNAs.

Bacterial regulatory non-coding sRNAs are heterogeneous in length (50—300 nt), and mostly regulate the translation efficiency and stability of target mRNAs through short and imperfect base pairing. In phytopathogenic bacteria such as *Xanthomonas*, *Agrobacterium*, and *Pectobacterium*, non-coding sRNAs are responsive to stress and may regulate pathogenic development [46-50]. For instance, the non-coding RNAs sX12 (67 nt) and sX13 (105 nt) are important for *Xanthomonas campestris* pv. *vesicatoria* pathogenicity, and deletion of these loci attenuates the virulence of the pathogen. HrpX, a key regulator of the type III secretion system (T3SS), induces sX12. Moreover, deletion of sX13 led to reduced accumulation of HrpF, HrcN, and HrcJ, suggesting that both of these non-coding RNAs contribute to virulence by regulating expression of or being regulated by the T3SS components [49,51]. Bacterial non-coding RNAs generally operate through conserved RNA-binding protein complexes, such as Hfq, CsrA/RsmA or CRISPR-Cas to execute gene regulation. It is very likely that such RNA-directed silencing complexes contribute to pathogenesis. Supporting evidence includes the findings that the *hfq* and *rsmA* deletion mutants exhibited attenuated virulence [52,53].

Diverse classes of sRNAs contribute to the pathogenesis of various eukaryotic pathogens as well [5,6]. In the rice blast fungus *Magnaporthe oryzae*, many sRNAs that are derived from tRNAs are enriched in appressoria, an infection-specific organ at the interaction interface [54]. A class of predominantly 24-nt sRNAs that mapped to long terminal repeat (LTR) retrotransposons is up-regulated during invasive growth [55]. The aggressive fungal pathogen *B. cinerea* can infect more than 200 plant species, including almost all vegetables and fruits. It produces a class of 21 to 22 nt sRNAs that mostly map to LTR retrotransposons. Some of these 21 to 22 nt *B. cinerea* sRNAs (Bc-sRNAs) are induced during the early stage of the infection process and target host genes from both *Arabidopsis* and tomato by hijacking host sRNA machinery [16] (Figure 1B). Microbial sRNA-induced host gene silencing is a naturally occurring cross-kingdom RNAi event utilized by aggressive pathogens as a novel virulence strategy. This study has added sRNAs to the list of pathogen effectors, molecules that are secreted and delivered into host cells to suppress host immunity.

A comparative study revealed that the three notorious oomycete *Phytophthora* plant pathogens, *P. infestans*, *P. sojae*, and *P. ramorum*, produce miRNAs as well as two distinct classes of siRNAs of 21 and 25 nt in length [56]. Several 21 nt siRNAs are generated in

antisense orientation to LTR retrotransposon loci. Many RXLR and CRN effector genes, crucial factors of *Phytophthora* infection and host adaptation, are located in close vicinity (< 2 kb) to LTR retrotransposons. RXLR and CRN effectors are specifically induced during the infection process. Interestingly, siRNAs were found to co-regulate retrotransposons and the surrounding CRN effectors [57,58], which impacts the interaction between the host and the pathogen [59]. How the production of effector gene-related siRNAs is regulated is currently unclear.

In summary, non-coding RNAs of bacterial plant pathogens and diverse classes of sRNAs in fungal and oomycete plant pathogens play pivotal roles in pathogen development, pathogenesis, and host specificity/adaptation (Figure 1A). It is important to understand the evolutionary processes and circumstances that are shaping the repertoires and expression levels of sRNAs in diverse phytopathogens as part of their virulence. Of particular interest is sRNAs that are capable of inducing silencing of host genes, as discussed below.

Cross-kingdom RNAi and sRNA trafficking

Cross-kingdom RNAi has been observed in both animal and plant systems. Plants transfer RNAi signals into interacting organisms, such as filamentous fungi, oomycetes, nematodes, parasitic plants, and pests [14,60,61], to suppress their growth in a process referred to as HIGS, the most prominent example of cross-kingdom RNAi in plants. Similar observations have been made in humans [62]. RNAi signaling in the opposite direction has also been reported. Recent discoveries showed that advanced pathogens and parasites use cross-kingdom RNAi to suppress host immunity for infection, as previously reviewed [3,12]. Such microbial sRNA-induced host gene silencing is not only present in plants as the case of *Botrytis*-host interaction, but it has also been observed in animal systems [15,63,64]. In addition, sRNAs produced by diverse parasites were observed in the body fluids of infected individuals [15,65-67], suggesting a common strategy of pathogens and parasites to secrete RNAi signals during infection to manipulate host cell immunity.

In plants, *B. cinerea* delivers some of its sRNAs into the host cells during early infection, and the predicted host targets of these Bc-sRNAs are highly enriched with signaling and regulatory genes. Three Bc-sRNAs are confirmed to suppress *Arabidopsis* and tomato immunity genes *in vivo*. Transgenic *Arabidopsis* lines ectopically expressing Bc-siRNAs show elevated susceptibility toward *B. cinerea* infection. Those Bc-siRNAs that target host genes share common features to plant miRNAs that are 21-22 nt in length with a 5’ first nucleotide uracil, which are preferentially loaded into *Arabidopsis* AGO1 for host gene suppression (Figure 1B). Consistent with this result, the *ago1-27* mutant, but not *ago2-1* or *ago4-2*, exhibit enhanced resistance toward *B. cinerea*. Interestingly, *ago1-27* also showed enhanced resistance against another fungal pathogen, *Verticillium dahliae* [38], even though most of the other *Arabidopsis* sRNA pathway mutants showed enhanced susceptibility. Whether pathogenic *Verticillium* spp. has evolved similar strategies to hijack host AGO1 to suppress host immunity, has yet to be determined. Furthermore, it is worthwhile to investigate how widespread this strategy is to use sRNAs as effector molecules to suppress host immunity or manipulate host physiology and whether it is present in symbiotic relationships.

Strikingly, recent studies in animal systems indicate that such manipulation strategies via cross-kingdom RNAi have also evolved in animal parasites [15]. The gastrointestinal nematode *Heligmosomoides polygyrus* and the filarial nematode *Litomosoides sigmodontis* secrete miRNAs via extracellular vesicles [15], which can be internalized by host cells to suppress host genes efficiently. It is currently unclear whether these miRNAs function through host AGO proteins. Remarkably, the strongest suppressive effect of these parasites' miRNAs was found on host gene *Dusp1*, which is targeted by three nematode miRNAs [15]. Dusp1 is an important regulator of MAPK pathways in animal systems. The plant pathogen *B. cinerea* has evolved to manipulate host *MAPK*s in *Arabidopsis* and a *MAPKKK* in tomato using mobile Bc-sRNAs. MAPK cascades are essential regulatory pathways in plant and animal immune signaling, and it is obvious that members and regulators of MAPK pathways are favorably targeted and manipulated by pathogen and parasite effectors including sRNAs (Figure 1B). Secreted sRNAs of diverse parasites have been identified in various hosts, including miRNAs of *Schistosoma japonicum* in plasma of infected rabbit [65], tRNA-derived sRNAs of *Trypanosoma cruzi* in infected susceptible mammalian cells [66], and miRNAs of *Onchocerca ochengi* and *O. volvulus* in nodule fluid of cattle, and plasma and serum of infected humans [67], respectively. These findings point to a common strategy of manipulating host immunity through secreted sRNAs.

Clearly, sRNA-guided cross-kingdom RNAi has evolved as an advanced virulence strategy adapted by pathogens and parasites of both plant and animal hosts. Furthermore, cross-kingdom gene silencing has been also found to act in an opposite direction in the case of HIGS (Figure 1B). Scientists have engineered diverse plant species, from model plants to commercial crops, to express exogenous artificial RNAi signals that target mRNAs of parasitic nematodes, herbivores, and fungal and oomycete pathogens for gene suppression, with the ultimate goal to create pest- and pathogen- resistant crops [13,68-70]. HIGS has yielded astonishing effects in enhancing plant resistance against a variety of pathogens and pests [60,61,71], demonstrating that it is an efficient strategy for crop protection. HIGS is also functional against parasitic plants, such as *Orobanche* and *Cuscuta* spp. [72]. Incredibly bidirectional transfer of thousands of mRNAs has been documented between the parasitic plant, dodder (*Cuscuta pentagona*), and two hosts, *Arabidopsis* and tomato [73-75]. Furthermore, RNA translocation between host and parasite is highly selective, as the profiles of the transferred parasite mRNAs and the total mRNAs within the parasitic plants were rather different. Although not examined, we speculate that sRNAs are also likely to be exchanged between the parasitic plant and its host.

In humans, resistance to the malaria pathogen *Plasmodium falciparum* is seen in people with sickle-cell anemia disease [76]. A recent study indicates that infected sickle cell erythrocytes overproduce certain miRNAs, which translocate into the *Plasmodium* cells and bind to *P. falciparum PKA-R* mRNA and block its translation [62], even though *P. falciparum* lacks the canonical RNAi pathway. This study demonstrates that animals have also evolved the cross-kingdom gene silencing strategy to suppress virulence of parasites. Examples of pathogen-induced host gene silencing, the cross-kingdom RNAi in the opposite direction, have been recently documented. However, what is the exact form of the mobile silencing signals and how these signals translocate from a host into the pathogen and parasite or *vice*

versa, is still poorly understood. The uptake of external RNAi signals that induces silencing in nematodes and insects (called environmental RNAi) has been well characterized, and nematode specific transporters were identified [69], although no obvious homologues of these transporters are found in other systems. Silencing of the cotton bollworm (*Helicoverpa armigera*) mono-oxygenase gene *CYP6AE14* by HIGS led to impaired tolerance to gossypol in larvae. HIGS against the cotton bollworm was still effective in an *Arabidopsis dcl2dcl3dcl4* mutant background that was unable to process the long dsRNA precursors of the *CYP6AE14* RNAs into mature siRNAs [77], which suggests that insects are likely to take up long dsRNA precursors. This is supported by findings of Zhang et al. [78], where long double-stranded plastid RNA was sufficient to induce gene silencing in pests.

Secretory pathways in plants, such as the exocytosis and the unconventional secretion pathway [79,80], as well as cellular uptake pathway of environmental substances, such as endocytosis [81,82], have been extensively studied. Secretion and uptake of proteins and other micro- and macromolecules is a hallmark of plant-microbe interactions and play key roles in plant defense against pathogens and parasite [83,84] and in pathogenesis and effector-triggered suppression of host plant immunity [83,85] However, secretion of RNAs, a known feature of cell-to-cell communications in animal systems [10,86], has hardly been documented in plants. We propose that plant export “channels” for RNAi triggers are not only sufficient for movement of artificial transgenic HIGS sRNAs; but it is most likely that some host endogenous sRNAs are also transported into pathogen cells for gene regulation (Figure 1B).

Conclusions

Plant host endogenous sRNAs and pathogen-derived sRNAs play pivotal roles in regulating cellular stress responses and plant immunity. Diverse classes of immune-regulatory sRNAs that are differentially regulated upon pathogen attack have been identified. Some of them are capable of translocation into interacting organisms to induce cross-kingdom RNAi. Hosts produce RNAi triggers, to silence pathogen/parasite genes, while advanced eukaryotic pathogens/parasites secrete sRNAs that mimic host sRNAs to suppress host immunity. Although cross-kingdom RNAi has been shown in many examples as HIGS, the mobile RNAi triggers and the mechanisms and pathway(s) of RNA transport are not known. We expect that well-designed genetic, biochemical, and cell biology assays will shed light on the transport mechanisms of cross-kingdom RNAi signals.

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Research highlights

- Small RNAs regulate plant immune responses and pathogen virulence.
- Small RNAs can move between interacting organisms and induce cross-kingdom RNAi.
- Advanced plant pathogens use cross-kingdom RNAi to suppress host immunity genes.
- Host induced gene silencing allows crops produce small RNAs silencing pathogen genes.

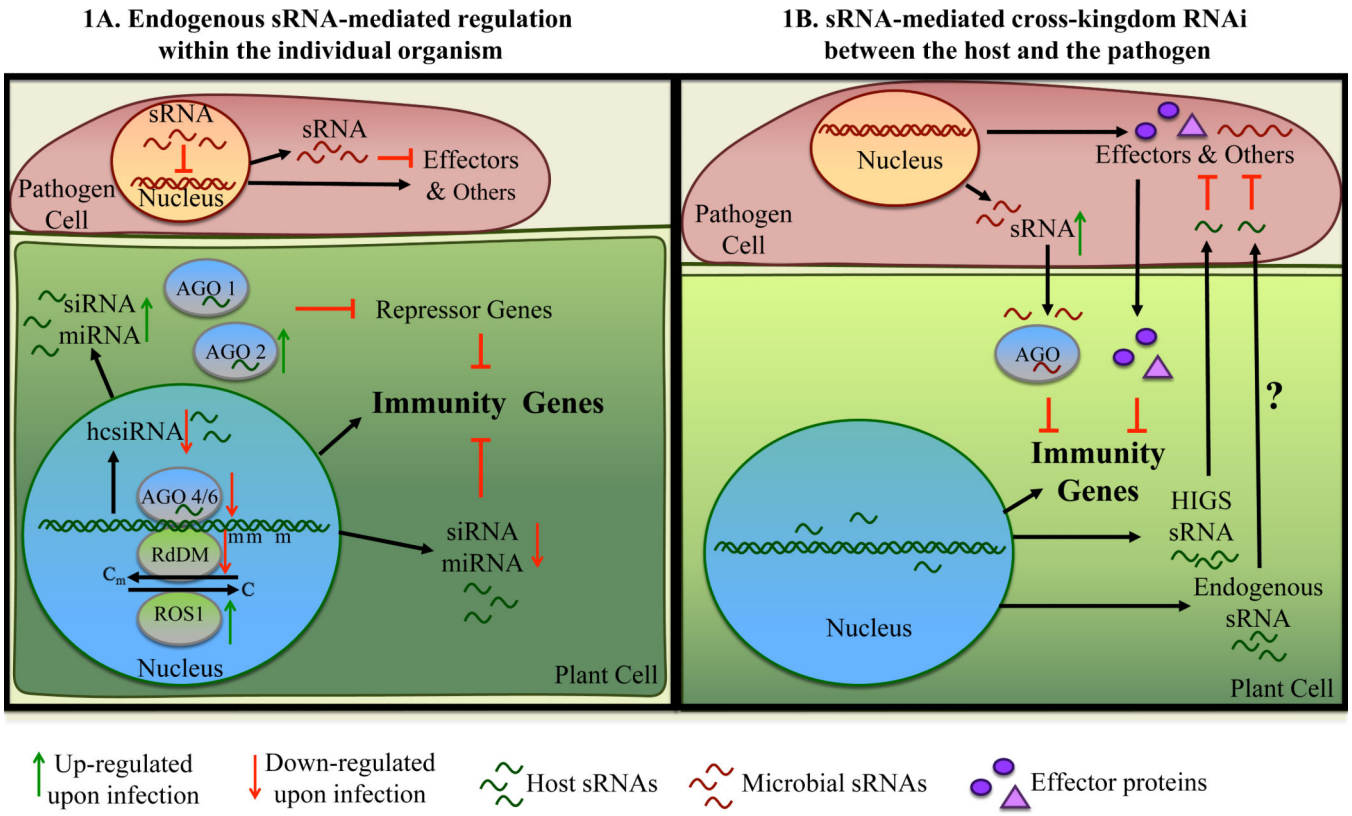

Figure 1.

sRNA-mediated regulation in plant-pathogen interactions

A) Plant and pathogen sRNAs regulate host immunity and pathogen virulence, respectively, within the individual organism via post-transcriptional and transcriptional gene silencing. In plants, miRNAs and siRNAs acting through AGO1 or AGO2 mediate post-transcriptional gene silencing; and transcriptional gene silencing is mediated by hcsiRNAs acting mostly through AGO4 and AGO6 to induce DNA cytosine methylation or histone modifications. In general, AGO2 is up-regulated, and AGO4/AGO6 are down-regulated by pathogen infection.

B) Plant and pathogen sRNAs trigger cross-kingdom RNAi in plant-pathogen interactions. Plant HIGS siRNAs and likely plant endogenous sRNAs target effector genes and other essential genes in pathogens, whereas pathogen sRNAs translate to host cells, and target host immune-responsive genes through the host AGO-RISC.