Published in final edited form as:

Curr Opin Plant Biol. 2017 August ; 38: 133–141. doi:10.1016/j.pbi.2017.05.003.

Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre- and post-harvest plant protection

Ming Wang, Nicholas Thomas, and Hailing Jin

Department of Plant Pathology & Microbiology, Center for Plant Cell Biology, Institute for Integrative Genome Biology, University of California, Riverside, California 92521-0122

Abstract

Small RNA (sRNA)-induced RNA interference (RNAi) is an important conserved mechanism that modulates gene expression in almost all eukaryotes. Some sRNAs move short distances from cell to cell, while some travel long distances to spread systemically throughout the organism. Recent studies indicate that sRNAs can even move between organisms to induce gene silencing, a phenomenon called “cross-kingdom RNAi”. sRNA trafficking between a pathogen, pest, or symbiont and its respective host can have a significant impact on interaction compatibility. Certain sRNAs were found to travel from pathogens or pests into host cells and suppress host immunity to achieve successful infection in both plants and animals; while sRNAs generated from host cells also translocate into pathogen or parasite cells to inhibit their virulence. Such cross-kingdom RNAi mechanisms enable the development of efficient disease control methods using plant-derived RNAs that target essential genes of pathogens and pests. Moreover, uptake of exogenous RNAs from the environment was recently discovered in certain fungal pathogens, which makes it possible to suppress fungal diseases by directly applying pathogen-targeting RNAs on crops and post-harvested products to avoid extensive chemical treatment and circumvent generating genetically modified plants. This spray-induced gene silencing (SIGS) strategy is environmentally sustainable and friendly, and can be easily adapted to control multiple fungal diseases simultaneously.

Introduction

Crop plants are constantly under attack by many pathogens and pests during both pre- and post-harvest stages, causing devastating food and economic losses worldwide. It has been estimated that in the field, microbial pathogens and pests cause up to 14.5% and 15.1% crop yield losses, respectively, despite extensive use of fungicides and pesticides [1]. Although it has been largely overlooked, destruction caused by post-harvest diseases during processing, transportation, and storage account for 20–25% crop reduction in the United States and up to 50% in some developing countries [2,3]. Current practices to control these diseases rely heavily on chemical treatments, which pose a serious threat to human health and the

Correspondence to: Hailing Jin.

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environment. Therefore, there is an urgent need to develop a more effective, sustainable, and environmentally friendly means to protect crops from pathogens and pests before and after harvest. This review describes the current understanding of cross-kingdom RNAi and RNA trafficking between pathogens/pests and their interacting hosts and environmental RNAi in fungal pathogens, as well as how these findings can be developed disease control methods.

Small RNAs and small RNA trafficking within an organism

Small RNAs (sRNAs) are short, non-coding regulatory RNAs that silence genes with appropriate base complementarity. sRNAs are generated from double-stranded RNAs (dsRNAs) or single-stranded RNAs with stem-loop structures by the RNase III-type endoribonucleases, Dicer or Dicer-like (DCL) proteins. sRNAs are loaded into Argonaute (AGO) proteins to silence target genes by RNA interference (RNAi) [4,5]. RNAi is a conserved eukaryotic gene regulatory mechanism that affects almost every biological process within an organism.

Some sRNAs are mobile and induce silencing of target genes non-cell autonomously. Within an organism, selective sRNAs were observed to move short distances from cell to cell or move systemically throughout the organism. Plant sRNAs move into neighboring cells most likely through the intercellular “bridge” plasmodesmata [6,7] or systemically via the phloem vascular structure [8-11]. Some sRNAs, including the 24 nt long heterochromatic sRNAs, move through grafting junctions via vasculature [8]. Although animal cells lack plasmodesmata, they have similar “gap junction” structures for intercellular connections that are responsible for sRNA trafficking between adjacent cells [12-14]. For example, human macrophages transfer endogenous microRNAs (miRNAs) to hepato-carcinoma cells mostly through gap junctions [15]. In *C. elegans*, a transmembrane protein systemic RNA interference defective-1 (SID-1) acts as a similar channel for intercellular RNA movement [16,17]. Several other SID proteins that contribute to systemic RNAi and sRNA movement were identified [18,19]. However, the proteins found in *C. elegans* that are responsible for sRNA movement are mostly specific to *C. elegans* and other nematodes [16,18,19], share no or very limited homologies to proteins in other species. In addition, several other RNA trafficking pathways between animal cells have been reported. Human sRNAs are transferred with AGO2 protein [20,21], high-density lipoprotein (HDL) lipid complexes [22] or extracellular vesicles (EVs). EVs are extracellular membranous vesicles that range from 40 to 1000 nm in diameter, which can be divided into two classes, including exosomes and ectosome (also called microvesicles, shedding vesicles, or microparticles), based on their size and origin [23,24]. Exosomes and ectosomes are derived from late endosomal membrane and plasma membrane, respectively [23,24]. They enable intercellular sRNA movement in animals, which also allows systemic spread of sRNAs in circulatory fluids [25-29]. Some miRNAs are transferred through apoptotic bodies following donor cell death [30]. Furthermore, extracellular miRNAs found in blood plasma and cell culture are predominantly associated with AGO proteins and are independent of vesicles.

Cross-Kingdom sRNA trafficking from pathogens and parasites into their hosts

In addition to sRNA trafficking within an organism, recent studies indicate that sRNAs can move between a host and interacting pathogens or parasites to induce gene silencing through a phenomenon called “cross-kingdom RNAi” [31,32].

Some plant and animal pathogens and pests are capable of delivering sRNAs into host cells to modulate host immune responses [33,34] (Figure 1). For example, the filamentous plant fungal pathogen *B. cinerea*, which causes grey mold disease on almost all vegetables, fruits, and flowers, has evolved an aggressive virulence mechanism using cross-kingdom RNAi [35]. Upon infection, *B. cinerea* delivers a group of sRNAs into host plant cells. These transferred sRNAs are loaded into the host *Arabidopsis* AGO1 protein to silence host immunity genes, such as mitogen activated protein kinases (MAPKs) and a cell wall associated kinase, etc [35-37]. It has been long known that pathogens deliver effectors, mostly proteins, into host cells to suppress host immunity [38]. These sRNAs from *B. cinerea* function as a novel class of pathogen effectors [35,37], which have the characteristic features that allow them to be loaded into host *Arabidopsis* AGO1 protein. Consistent with this finding, *B. cinerea* causes much less disease symptoms on the *Arabidopsis ago1-27* mutant compared to wild type plants because sRNA effectors could no longer function without host AGO1 [35,37]. Less severe disease symptoms were also observed on *Arabidopsis ago1-27* mutants infected with another fungal pathogen, *Verticillium dahliae*, which causes Verticillium wilt disease on many plants [39,40]. In agreement with this notion, *V. dahliae* sRNAs that have potential host targets are more predominantly associated with *Arabidopsis* AGO1 than AGO2 during infection [40]. These studies suggest *V. dahliae* also uses sRNAs as effectors to silence host target genes through *Arabidopsis* AGO1. Furthermore, the *B. cinerea dcl1 dcl2* double mutant strain that fails to produce sRNA effectors is compromised in pathogenicity on various plant species, including vegetables, fruits, and flowers, as compared to the wild type *B. cinerea*; whereas *B. cinerea dcl1* or *dcl2* single mutant, which exhibits significant growth defect on plates and on plants but can still produce sRNA effectors, still maintain aggressive virulence, supporting that sRNA effectors are essential for *B. cinerea* pathogenicity [35,40].

Similar observations of cross-kingdom sRNA trafficking were made also in animal systems [41-45]. For example, the gastrointestinal nematode *Heligmosomoides polygyrus* delivers sRNAs into mice gut epithelial cells [45]. It has been shown that exosomes, a distinct type of EVs, are largely responsible for sRNA trafficking between cells or systemically within an animal organism [25-29]. Buck et al. demonstrated that *H. polygyrus* exosomes are also responsible for delivering miRNAs into host cells to suppress host inflammation and immunity genes, including regulators of MAPK pathways [45]. Animal parasites secrete exosomes that contain sRNAs, which are internalized into host cells and likely silence host target genes [41-45]. Moreover, exosomes protect sRNAs from degradation by RNases in body fluids, which explains why the sRNAs remain stable and active after traveling into host cells [42,45]. Whether EV-mediated sRNA trafficking is a general pathway for sRNA transfer from pathogens/pests to hosts is unknown. Various RNA molecules, including

sRNAs, are present in EVs isolated from human pathogens *Cryptococcus neoformans*, *Paracoccidioides brasiliensis* and *Candida albicans*, and from the model yeast *Saccharomyces cerevisiae* [46]. It is possible that EVs mediate RNA export and communication with host cells during infection. Apart from EV-mediated trafficking, some proteins evolved to facilitate RNA trafficking in certain organisms, as in the case of *C. elegans*, which is discussed in the environmental RNAi section below.

Communications through sRNAs and cross-kingdom RNAi may exist between many interacting organisms, including those with a beneficial symbiotic relationship [47]. In fact, genome sequencing analysis has revealed a set of miRNAs of the dinoflagellate symbiont *Symbiodinium kawagutii*, an important photosynthetic endosymbiont of coral that can potentially target its own genes and coral host genes. The predicted host target genes have similar putative functions as the endogenous *S. kawagutii* miRNA targets, suggesting that these mobile miRNAs may modulate similar biological processes in both host and symbiont [47].

Interestingly, such RNA movement to host cells is not limited to eukaryotic pathogens or pests. Prokaryotes do not have RNAi machinery, they produce small non-coding RNAs that are 70-200 nt in length. In fact, in the endosymbiotic host-bacteria interactions, two bacterial small non-coding RNA WsnRNA-46 and WsnRNA-49 from *Wolbachia*, a symbiotic bacterium infects many arthropod species, can regulate the target genes from the mosquitoes host *Aedes aegypti* in addition to its own genes [48].

Cross-Kingdom sRNA trafficking from hosts to pathogens or parasites

Host-induced gene silencing (HIGS) is an excellent example of sRNA trafficking from hosts to interacting pathogens or pests and has been extensively investigated during the last decade for as a method of crop protection [49-53]. Transgenic plants and crops expressing sRNAs that target essential growth and virulence genes of eukaryotic pathogens and pests are resistant/tolerant to disease [49-53] (Figure 1). For example, cotton bollworm larva fed plant material expressing dsRNAs that target and reduce the expression of a cytochrome P450 gene are more sensitive to the anti-herbivory plant compound gossypol [52]. Moreover, transgenic barley plants expressing dsRNAs targeting the fungal pathogen *Blumeria graminis* development gene that encoding 1,3- β -glucanosyltransferase (*GTF1*) or effector genes *AvrA10* and *AvrK1* displayed significantly reduced disease symptoms caused by *B. graminis* [50]. Direct evidence that sRNA is transferred from host to pathogen in HIGS was provided by Wang et al. [40]. Host-derived *B. cinerea* *DCL1/2*-targeting sRNAs were identified in the interacting *B. cinerea* *dcl1 dcl2* mutant strain. Because the *dcl1 dcl2* mutant *B. cinerea* strain can no longer process dsRNA precursors into sRNAs, it eliminates the possibility that the sRNAs detected in *dcl1 dcl2* were processed by fungal DCLs [40]. Although various forms of RNAs may have the potential to move across organism boundaries, this result supports that sRNAs are at least one of the major mobile signals for cross-kingdom RNAi. Such bidirectional RNA trafficking was also observed in the interaction between an invertebrate host and its parasite. In the interaction between the honey bee (*Apis mellifera*) and its obligatory ectoparasite *Varroa* mite, ingested artificial dsRNAs are transferred from *A. mellifera* to *Varroa* and vice versa, triggering bidirectional

RNAi *in trans* [54]. These studies provide excellent examples of bidirectional cross-kingdom RNAi and sRNA trafficking between interacting organisms.

In addition to artificial transgene-derived sRNAs, animals and plants can also deliver endogenous sRNAs into interacting organisms [55,56]. In the interactions between human and malaria parasite *Plasmodium falciparum*, some erythrocyte miRNAs, including miR-451 and lethal-7i (let-7i), are translocated into *P. falciparum* [56]. Although *P. falciparum* lacks RNAi machinery, these transferred miRNAs utilize an alternative mode of action by forming chimeric transcripts fused to parasite target mRNAs, *cAMP-dependent Protein Kinase subunit (PKA-R)* and *reduced expression 1 (REX1)*, and inhibit mRNA translation of these pathogenicity related genes [56,57]. This phenomenon may explain why patients with sickle cell anemia are more resistant to malaria, because they have elevated levels of these *P. falciparum* gene-targeting miRNAs [56]. In cotton, the abundant miR166 and miR159 were recently found to move into *V. dahliae* hyphae and down regulate two *V. dahliae* target genes encoding a Ca²⁺-dependent cysteine protease (Clp-1) and an isotrichodermin C-15 hydroxylase (HiC-15), respectively [55]. Both Clp-1 and HiC-15 play an important role in the pathogenicity of *V. dahliae*. These studies suggest that plant and animal hosts also utilize cross-kingdom RNAi strategies to suppress the virulence of pathogens and parasites.

Strikingly, hosts can also deliver sRNAs into prokaryotic pathogens. In the interactions between mammalian hosts and gut bacteria, host-derived fecal miRNAs enter the gut bacterial cells and co-localize with bacterial nucleic acids to regulate transcript level of the targets, thus affect the growth of gut bacteria [58]. For example, the human miRNAs hsa-miR-1226-5p and hsa-miR-515-5p enter the gut bacteria *Escherichia coli* (*E. coli*) and *Fusobacterium nucleatum* (*Fn*). Intriguingly, these host miRNAs increase the transcript level of their bacterial targets *E. coli yegH* and *Fn* 16s rRNA, respectively [58]. Thus, these host fecal miRNAs promote the growth of gut bacteria.

It remains to be determined how common it is for hosts to suppress disease by delivering sRNAs into interacting pathogens and pests to manipulate virulence genes. The fact that RNA trafficking-mediated defense mechanisms are present in both plant and animal hosts supports the important role of cross-kingdom RNAi in the evolution of host immune responses.

Environmental RNAi and spray-induced gene silencing

HIGS has been demonstrated to be successful in plant protection against nematodes [51], insects [52,53], fungi [40,50,59], and oomycetes [60,61]. Such sequence-based cross-kingdom RNAi strategies could be easily adapted to control multiple pathogens simultaneously by targeting essential virulent genes from different pathogens (Figure 1) [40]. Transgenic plants expressing sRNAs that target essential virulence genes *DCL1* and *DCL2* from both *B. cinerea* and *V. dahliae* exhibit enhanced resistance/tolerance to both fungal pathogens [40]. One of the limitations of HIGS is that it requires stable genetic transformation, which is not yet possible for many economically important crops. Additionally, the public still has concerns about genetically engineered crops, commonly

known as genetically modified organisms (GMOs). It is highly desirable to develop new means of disease control without generating GMOs or extensive use of chemicals.

Uptake of external RNAs from the environment that induce RNAi, a phenomenon called “environmental RNAi”, was observed in *C. elegans* and several nematodes and insects [62]. Mutant screenings in *C. elegans* identified several *SID* genes that are responsible for environmental RNAi and systemic RNAi [16-19,63,64]. However, most of these genes are almost exclusively present in invertebrates but not in fungi or plants [34]. For example, *SID-1* and *SID-2* are two important genes that mediate dsRNA uptake. *SID-1* encodes a transmembrane protein that serves as a channel for rapid and endocytosis-independent dsRNA uptake [16,17], while *SID-2* is a transmembrane protein that mediates slow and endocytosis-dependent dsRNA uptake [19,63]. *SID-1* has a very distant homolog in mammals but not in *Drosophila*, plants, or fungi [16], and *SID-2* only has homologous genes in two closely related nematodes, *C. briggsae* and *C. remanei* [19].

So far, environmental RNAi has not yet been observed in mammals. It was not clear whether plants and fungi could take up RNAs from the environment until recently. Wang et al. recently demonstrated that the fungal pathogen *B. cinerea* is capable of taking up dsRNAs and sRNAs from the environment (Figure 1) [40]. This RNA uptake makes it possible to use dsRNAs or sRNAs that target pathogen genes directly for disease management. Indeed, spraying *B. cinerea* *DCL1/2*-targeting dsRNAs or sRNAs on the surface of fruits, vegetables, and flowers significantly inhibits grey mold diseases [40]. Spray-induced gene silencing (SIGS) is also effective for disease control in monocots [65], which represent some of the most important crop species (Figure 1). Koch et al. demonstrated that spraying dsRNAs that target *Fusarium graminearum* cytochrome P450 lanosterol C-14 α -demethylase (*CYP51*) genes significantly reduced disease symptoms on barley leaves [65]. Thus, these RNAs that targeting pathogen genes serve as a new generation of fungicides that are effective for both dicot and monocot crop species [66].

External RNAs may be translocated into pathogens/pests by either direct or indirect mechanisms (Figure 1). RNAs could either be directly taken up by the fungal cells or be transferred into plant cells first then move into fungal cells [40,65]. Interestingly, locally sprayed RNAs also inhibit pathogen virulence at distal, non-sprayed leaves [65,67] suggesting that these RNAs are able to spread systemically within plants. It is worth noting that invertebrates typically take up dsRNAs that are longer than 50 bp in length but not shorter dsRNAs or sRNAs [17,68,69], while fungi and plants can take up both dsRNAs and sRNAs [40,65], suggesting that the invertebrates may share different uptake mechanisms compared to fungi or plants.

SIGS has also been used to control insect pests. Topically applying synthesized dsRNAs that target insect developmental genes significantly increases the rate of mortality and impairs insect growth [70-73]. Such an effect can also be achieved by irrigating plant roots with dsRNAs targeting insect genes, which efficiently leads to target gene silencing and abnormal development of the pest insects [74]. Interestingly, high mortality rates of the pest insect *Plutella xylostella* were observed on plant leaves sprayed with sRNAs targeting the insect *P. xylostella* acetylcholine esterase genes *AChE1* and *AChE2* [75]. These results support that

gene expression within insect pests are suppressed through uptake of either dsRNAs or sRNAs.

Recent advances in nanoparticle technology have improved the potential applications of SIGS for plant protection. Naked dsRNA and sRNA treatments can protect plants from microbial pathogens up to 10 days after spraying [40,65]. A recent study indicates that the duration of protection against viral infection was extended to more than 20 days when dsRNAs were incorporated into layered double hydroxide (LDH) clay nanosheets called BioClay [67]. BioClay nanosheets prevented dsRNAs from being degraded by RNases or sunlight, or from being easily washed away from leaf surfaces by water. In fact, nanoparticles, including LDH nanoparticles, have been widely used in RNA delivery to facilitate RNAi in human therapies [76,77]. Because these nanoparticles and the RNAs within them are non-toxic and easily degradable, this technique is an environmentally conscious method that improves the efficacy of plant disease management in the field using SIGS.

SIGS provides safe and powerful plant protection not only on pre-harvest crops [65,67] but also on post-harvest products [40]. Fruits, vegetables, grains, and decorative plants succumb to post-harvest attack by microbial pathogens during processing, transportation and storage [2]. Furthermore, pathogens often produce toxic chemicals while proliferating on post-harvest products. For example, fungal pathogens such as *Aspergillus*, *Penicillium*, and *Fusarium* produce mycotoxins on post-harvest grain products. Mycotoxins are considered carcinogenic and can pose a serious threat to consumers' health [78]. Currently, application of fungicides and microbial antagonists is still the most commonly used strategy for controlling post-harvest diseases. Therefore, controlling post-harvest diseases using a new generation of sustainable and environmentally friendly RNA-based fungicides can help reduce the yield loss caused by post-harvest damage as well as prevent the accumulation of toxic chemicals produced by pathogens.

Conclusion

The emerging evidence on cross-kingdom RNAi and RNA trafficking has expanded our knowledge of host-pathogen interactions and potential disease management approaches. Such RNA exchange may be a common mechanism of communications present in many interacting organisms. More work is needed to understand the precise molecular mechanisms governing cross-kingdom RNA trafficking to better understand its evolution and how it shapes host-pathogen or host-pest interactions. Although isolating pure cell fractions of an individual organism from the interacting interface is still technically challenge, more and more endogenous mobile sRNAs have been identified in different organisms under different conditions [8,9,26,28,29,79,80], and their profiles are largely different from the profiles of total sRNAs. One future direction of sRNA research is to understand the molecular mechanism of mobile sRNA selection under different developmental and environmental conditions.

Simulating cross-kingdom RNAi using SIGS presents an attractive, powerful, and safe alternative to the current chemical-based applications for disease control [2]. Compared to

current disease control methods, SIGS is a more targeted and environmentally friendly strategy for both post- and pre-harvest plant protection and is less detrimental to consumer health. SIGS is also environmentally sustainable, because the possibility that pathogens will evolve resistance to these RNA-based fungicides is low, as SIGS is sequence-based and does not require 100% base-pairing for effective silencing, and many nucleotide mutations in the pathogen would be required to evade the targeting sRNAs. Additionally, since SIGS often chooses to target pathogen genes that are essential for growth or virulence, the pathogens may not be able to afford evolving enough mutations in these essential genes to evade SIGS yet while retaining their vital function. Finally, we believe that SIGS would be more acceptable to the public than the use of chemical treatments, and its development would take less time than generating stable transgenic crops or GMOs.

Acknowledgments

We apologize that we would not be able to include and cite many related interesting studies due to the limited space. The work in Jin's lab has been supported by grants from National Institute of Health (R01 GM093008), National Science Foundation (IOS-1257576, IOS-1557812) and an AES-CE Award (PPA-7517H).

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Highlights

- Small RNAs can transfer from pathogens or parasites into their interacting hosts.
- Plant and animal hosts can deliver small RNAs into interacting pathogens and parasites.
- External RNAs can be taken up by fungal cells and plant cells and induce RNAi.
- Spray-induced gene silencing represents an innovative disease control tool.

Figure 1. Cross-kingdom RNAi and spray induced gene silencing are effective strategies for preventing pre- and post-harvest diseases

This schematic illustrates the movement of RNAs between plant fungal pathogens and their hosts and how spray-induced gene silencing (SIGS) can be used to counteract pathogen virulence. Pathogen-derived sRNA effectors are delivered into the host, where they suppress host immune responses (red and blue block arrows). The spray application of gene-specific RNAs can suppress virulence through RNA interference in multiple pathogens (red and blue block arrows) either on crops or post-harvest products. These RNAs can either translocate directly to the eukaryotic pathogen or indirectly through the host (red and blue arrows). SIGS-based protection can be prolonged by incorporated RNAs into clay nanosheets (in grey) that protect RNAs from degradation and from being washed away. These RNAs can also spread systemically between cells or to other tissues in the plant (purple arrows), most likely through plasmodesmata and vascular phloem structures. The background represents the variety of crops and post-harvest products in which SIGS can be used to prevent loss from disease.