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Conversations between kingdoms: small RNAs

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Humans, animals, and plants are constantly under attack from pathogens and pests, resulting in severe consequences on global human health and crop production. Small RNA (sRNA)-mediated RNA interference (RNAi) is a conserved regulatory mechanism that is involved in almost all eukaryotic cellular processes, including host immunity and pathogen virulence. Recent evidence supports the significant contribution of sRNAs and RNAi to the communication between hosts and some eukaryotic pathogens, pests, parasites, or symbiotic microorganisms. Mobile silencing signals — most likely sRNAs — are capable of translocating from the host to its interacting organism, and vice versa. In this review, we will provide an overview of sRNA communications between different kingdoms, with a primary focus on the advances in plant–pathogen interaction systems.

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Introduction

Cell-to-cell communication occurs between organisms that form pathogenic, parasitic, or symbiotic relationships. Such communication involves transportation of regulatory molecules across the cellular boundaries between the host and its interacting pathogens/pests/parasites or symbionts. Recently, mobile small RNAs (sRNAs) have been indicated to function in communication between hosts and advanced pathogens/pests/parasites. sRNAs are non-coding regulatory RNAs that are loaded into Argonaute (AGO) proteins to silence genes with complementary sequences in a mechanism called RNA interference (RNAi). The RNAi machinery is conserved in most eukaryotes and mediated by non-coding small interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-associated RNAs (piRNAs). RNAi functions not only as a defense mechanism to silence foreign DNA and RNA species such as those from viruses, transposons, and transgenes, but also plays an important role in regulating and fine-tuning the expression of genes in a plethora of diverse physiological and cellular processes, including host immune responses [1–4]. Mobile, cell non-autonomous sRNAs that translocate within an organism have been observed in various plant [5–9] and animal systems [10–12,13•]. Some sRNAs can even move across the boundaries between hosts and their interacting pathogenic, parasitic, or symbiotic organisms and trigger gene silencing *in trans* in the non-related species, a mechanism termed cross-kingdom or cross-organism RNAi [4,14,15]. Here, we review the latest discoveries on cross-kingdom regulatory sRNAs with an emphasis on plant–microbial interactions and potential applications for mobile sRNAs in the future of plant biotechnology. sRNA-directed RNAi enriches the toolbox for plant researchers to manipulate gene expression to bolster plant resistance and, furthermore, to modulate the outcome of plant interactions with other organisms.

Mobile small RNAs

Mobile sRNAs, or possibly their precursor RNAs in certain conditions, which spread gene silencing into adjacent cells and tissues or even spread systemically, have fascinated scientists for the last two decades. From the time the phenomenon was first discovered [16–18], genetic determinants, pathways, and mechanisms have been revealed in a variety of organisms [5,7,19,20]. Diverse functions and useful applications for extracellular sRNAs have been established, encompassing cell-to-cell signaling and communication in multi-cellular organisms [13•], trans-generational RNAi [21,22] and memorization [23–26], cell fate differentiation and vascular formation [27–31], systemic antiviral immunity [32], environmental RNAi [11,33], cancer prevention and diagnosis [34], and intercellular immune activation [13•,35–37].

Cross-kingdom RNAi is a form of communication between two, often unrelated, interacting organisms such as a host and its pathogen, pest, parasite, or symbiont. This phenomenon has been overlooked in the past due to technical limitations. In respect to extracellular interacting organisms, such as bacteria, fungi, oomycetes, protozoa, nematodes, parasites, or herbivores, cross-kingdom RNAi implies that a translocation of gene silencing signals occurs between hosts and these organisms. These silencing signals may utilize conserved cell-to-cell as well as systemic RNAi pathways present in plants and animals, and may also use organism-specific pathways. The

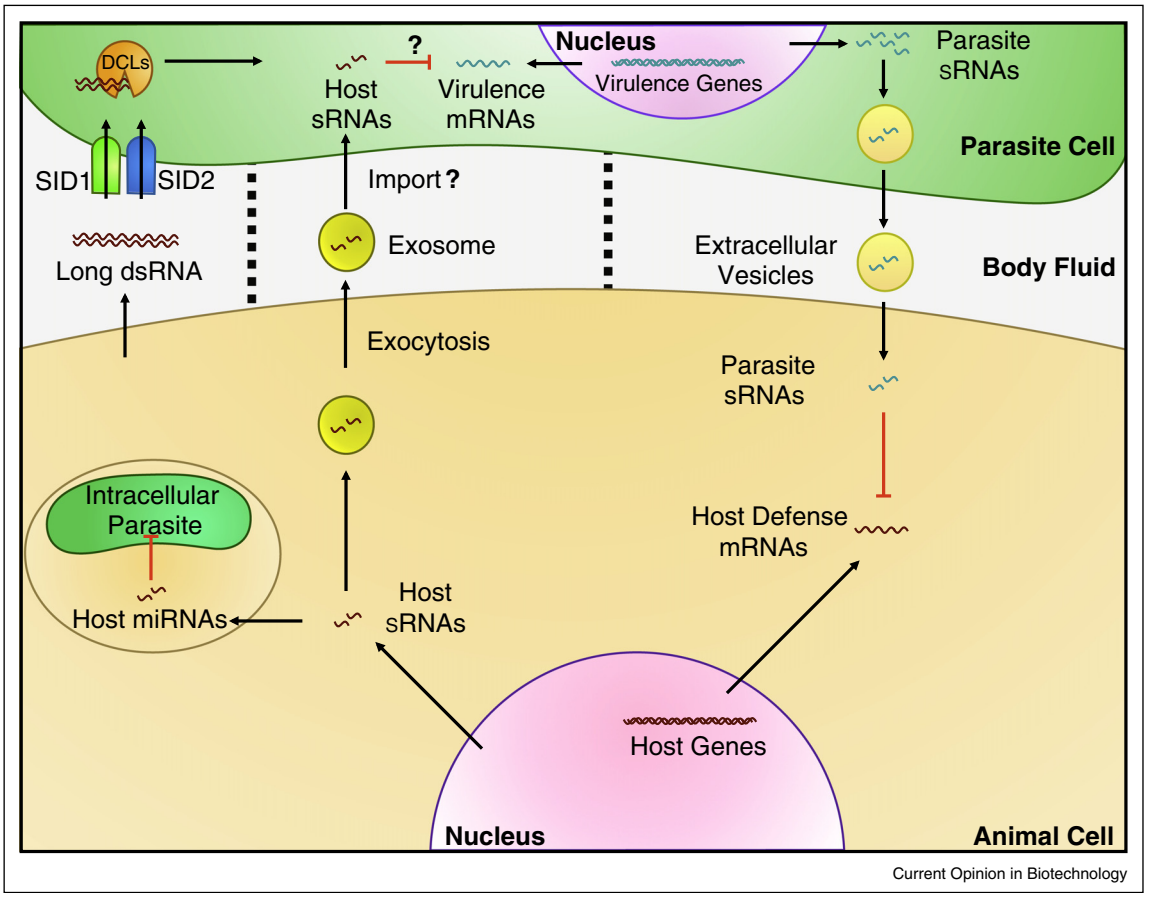
language of RNAi-based inter-species cross talk could be termed 'social RNAs' [38].

Cross-kingdom gene silencing in animal systems

There are few instances that point to the existence of cross-kingdom gene silencing in animal systems. One example is environmental RNAi in *Caenorhabditis elegans*, in which the worms uptake environmental RNA signals that have gene suppressive effects [11]. RNAi can be induced by soaking the worms in RNA solutions or by feeding them antisense RNA-expressing bacteria, such as *Escherichia coli*. A number of genes that are required for the uptake of environmental long dsRNAs as well as systemic silencing have been discovered in *C. elegans*, such as systemic RNAi defective-1 (*sid-1*) and *sid-2*, two transmembrane RNA transporters [19,20] (Figure 1). However, many identified transporter-like proteins are specific to worms or invertebrates. In a recent feeding experiment, two natural non-coding RNAs from *E. coli*, *OxyS* and *DsrA*, could suppress protein-coding genes in *C. elegans* [39••]. Gene suppression of the *che-2* mRNA (a WD-40 protein involved in chemosensory) by *OxyS* relied heavily on distinct RNAi genes, such as the AGO protein ALG-1, the dsRNA-binding protein RDE-4, and the ABC transporter HAF-2. The *sid-1* and *sid-2* mutants did not show any alteration in gene suppression, probably due to the redundant function of *OxyS* and *DsrA*-mediated gene silencing. *OxyS* is induced by oxidative stress, while its primary role is translational repression of *E. coli* mRNAs *rpoS* (sigma subunit of RNA polymerase) and *fhlA* (transcription activator). 'Why' and 'how' *E. coli* regulatory RNAs evolved to target genes from an unrelated species like *C. elegans in trans*, remains to be illustrated.

Cross-kingdom RNAi has also been observed in host–parasite interactions (Figure 1). The protozoan malaria

Figure 1



Cross-kingdom gene silencing between animal cell and parasites. Animal cells produce host sRNAs, and selected host miRNAs translocate into intracellularly phagocytized parasites and target parasitic mRNAs. Some mammalian sRNAs are selectively sorted into vesicles for secretion via exocytosis (exosomes). Extracellular parasite cells likely internalize exosomal sRNAs and, in addition, take up extracellular long dsRNAs via cell membrane-associated RNA transporters, SID-1 and SID-2. Long dsRNAs are further processed into mature sRNAs by DCLs; both pathways may trigger gene silencing in the parasite. Parasites encapsulate and secrete parasitic sRNAs that circulate in the body fluids of an infected individual and are internalized by host cells, triggering parasitic-induced host gene silencing.

parasite *Plasmodium falciparum* infects humans by entering the blood stream and multiplies intracellularly. It has long been known that individuals with sickle cell disease resist infection by *P. falciparum*, however the underlying mechanism was not fully understood. The dysregulated miRNA composition in these cells was recently found to contribute to this resistance. The erythrocytes infected by *P. falciparum* produce miRNAs that are translocated into the parasitic cells in high concentrations [40••]. Two highly enriched human miRNAs in erythrocytes of sickle cell individuals, miR451 and let-7i, were demonstrated to bind to *Plasmodium* mRNAs. One target gene of miR451 is the *cAMP-dependent protein kinase PKA-R*. Overexpression of miR451 and let-7i led to reduced parasitemia, suggesting that translocated human miRNAs suppress virulence-associated mRNAs in the parasite (Figure 1). It is worth noting that *Plasmodium* lacks essential RNAi components, such as AGOs and Dicer or Dicer-like (DCL) proteins that process the double-stranded RNA precursors into sRNAs, which suggests that the mode of action of sRNA-mediated gene suppression in this interaction may be independent of the canonical RNAi pathway. Binding of miR451 to *PKA-R* mRNA is likely to block ribosomal loading and causes translation inhibition [40••].

In mammals, cell-to-cell communication is mediated by exosomal vesicles that contain miRNAs (Figure 1) [13•]. Exosomal miRNAs have specific functions such as immune response activation [37]. The helminth nematode *Heligmosomoides polygyrus* also utilizes exosomal vesicles to increase virulence in a fashion similar to that of the mammalian miRNA transport mechanism [41••]. *H. polygyrus* secretes miRNA-loaded vesicles that are accompanied by a nematode AGO protein, most likely to stabilize the miRNAs. Remarkably, *H. polygyrus* vesicles are internalized by mice cells, which results in suppression of host immunity. Some *H. polygyrus* miRNAs were shown to target *in vitro* host mRNAs that are related to host immunity [41••]. However, it needs to be determined through which AGO protein — nematode AGO or host AGO — these nematode-derived miRNAs silence host genes. Taken together, nematode vesicles resemble their mammalian exosomal miRNA transport counterparts [42,43].

Evidence has shown that parasites can secrete sRNAs into their host during infection. sRNAs originating from parasites, such as protozoa *Trypanosomas cruzi* [44] and *Schistosoma japonicum* [45] and the nematode *Litomosoides sigmodontis* [41••], have been found in the body fluids of infected individuals, indicating that an invasion of circulating sRNAs in host systems may be a common event (Figure 1).

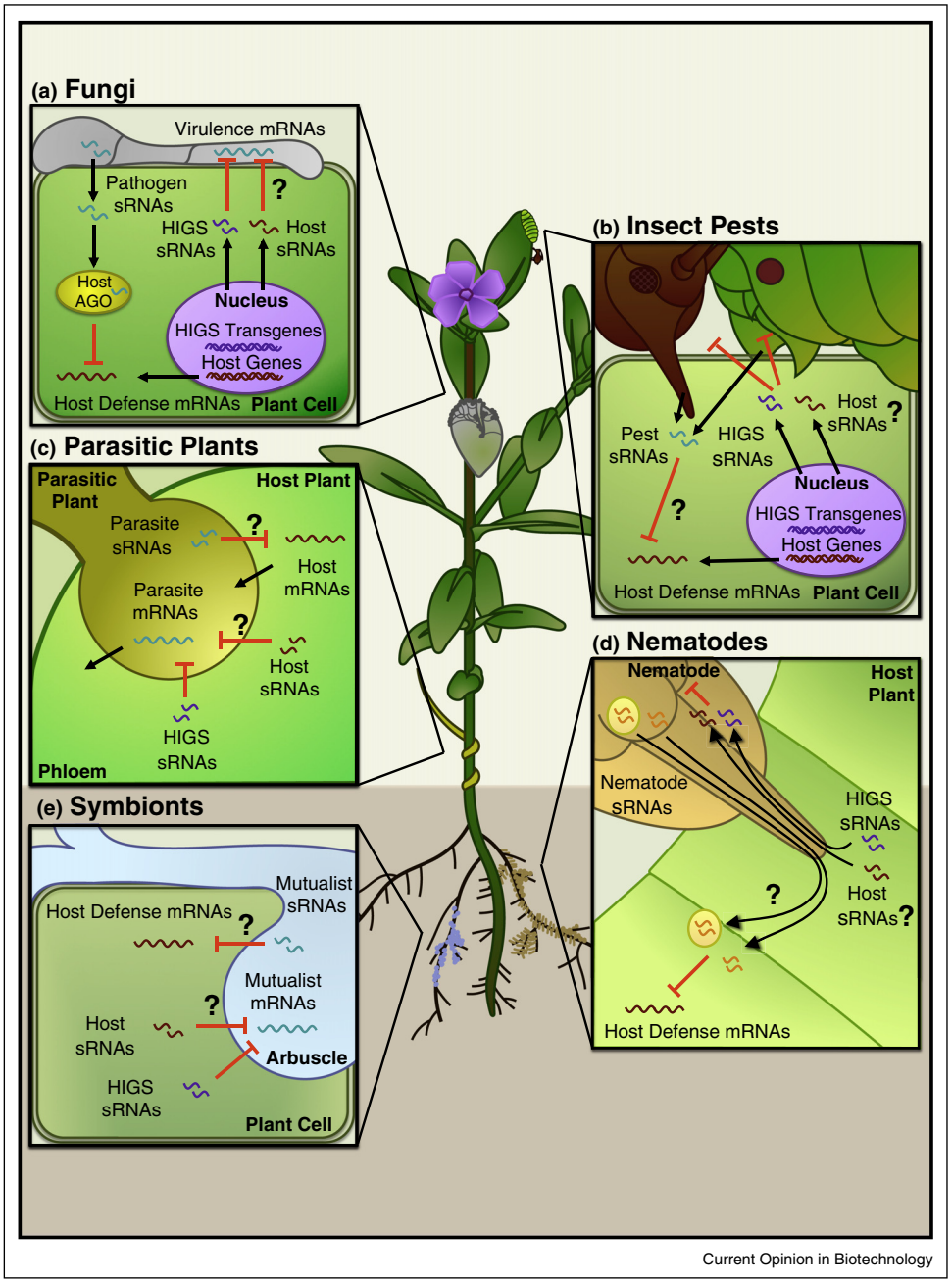
Plants communicate with their interacting organisms using mobile sRNAs

Because of their sessile nature, it is of vital importance that plants are in constant communication with their interacting organisms and environment. Host defense responses induced by pathogens/pests/parasites, or signal transduction triggered by the communication between hosts and symbionts, or communities of endophytic organisms, are all initiated by molecular signals. sRNAs, and possibly their precursor RNAs in certain situations, function as mobile signals that spread silencing information to influence the interacting organisms.

RNAi in fungi has been best studied in the model systems *Neurospora crassa* and the fission yeast *Schizosaccharomyces pombe* [46,47]; yet, neither species are natural pathogens or symbionts of plants. Fungal sRNA biogenesis pathways are diverse, and include both DCL-dependent and DCL-independent pathways [48,49]. Most eukaryotic microbes that come into intimate contact with plants, including pathogenic fungi and oomycetes, possess functional RNAi pathways and produce regulatory sRNAs. Remarkably, scientists have developed an effective disease control strategy, called host-induced gene silencing (HIGS) [50,51], by generating transgenic plants that express exogenous RNAi triggers to successfully silence essential genes in pathogens and pests. In addition to its successful use in model plants such as *Arabidopsis thaliana* and tobacco *Nicotiana benthamiana*, HIGS has been also successfully applied in important crops, including wheat, barley, *Medicago*, and banana, to efficiently work against a variety of fungal and oomycete pathogens, such as *Blumeria graminis*, *Puccinia tritici*, *Fusarium* spp., and *Phytophthora capsici* (Figure 2a) [51,52]. The use of HIGS to combat fungal pathogens caused alteration in fungal morphology, growth inhibition *in planta*, and most importantly, reduced virulence. In addition, HIGS is a powerful tool to study gene function in non-transformable species [50,53•]. A HIGS approach was applied to study gene function of the *Monosaccharide Transporter 2* from *Glomus* sp. [54•], demonstrating that HIGS is functional also on arbuscular mycorrhiza, which forms symbiotic relationship with hosts. The artificial sRNAs generated from host plants could be transported into arbuscular mycorrhiza and to be functional. Most surprisingly, silencing effects were also observed after external treatment of fungal mycelium with corresponding duplex sRNAs, indicating that a sufficient RNA uptake system must exist in fungi [55••].

The successful application of HIGS demonstrates the ability of plants to deliver mobile gene silencing signals to communicate with and manipulate diverse interacting organisms. However, some pathogen-produced sRNAs are capable of inducing gene silencing in the plants, too. A positive role of sRNAs in fungal virulence is supported by the fact that fungal sRNAs differentially accumulate during the infection process [56••,57]. Moreover, the aggressive fungal plant pathogen *Botrytis cinerea* produces sRNAs (Bc-sRNAs) that move into the host plant cell during early infection and hijack the host AGO,

Figure 2



Cross-kingdom gene silencing between plants and interacting organisms. Host-induced gene silencing (HIGS) sRNAs are produced via transgenes in plants. **(a)** HIGS sRNAs translocate into pathogens and suppress virulence mRNAs in pathogenic fungi and oomycetes. We speculate that there are natural host sRNAs that target virulence mRNAs for host defense via similar pathways. Pathogen-derived sRNAs that mimic host sRNAs are translocated into host cells to hijack the host AGO/RISC machinery to suppress host immunity mRNAs. **(b)** HIGS sRNAs are effective against insect pests by translocating into insect pest cells and silencing their virulence genes. Similarly, natural host sRNAs could also potentially target pest mRNAs for silencing. At the same time, pest sRNAs are also likely to be injected into host cells to suppress host immunity or manipulate other cellular pathways. **(c)** Parasitic plants, such as *Cuscuta* sp., form haustoria to acquire nutrients from the phloem source. Bi-directional exchange of a large array of mRNAs has been observed. It is likely that sRNAs are also transported between hosts and parasitic plants as gene regulators. **(d)** HIGS sRNAs are effective against plant-parasitic nematodes. It is possible that natural host sRNAs may target nematode mRNAs for defense. On the other hand, nematode-induced gene silencing of host mRNAs is also likely, either via encapsulated or non-vesicular nematode sRNAs. **(e)** HIGS sRNAs are effective against mRNAs from symbiotic organisms, such as mycorrhiza. Similarly, natural host sRNAs, as well as symbiotic sRNAs, are also likely to be exchanged during the regulation of symbiosis.

the key protein in the RNAi machinery, to silence important host immunity genes [56••]. This observation points to the bi-directional nature of cross-kingdom RNAi in plant–pathogen interactions (Figure 2a). Some Bc-sRNAs structurally mimic plant sRNAs that specifically bind to *Arabidopsis* AGO1 (AtAGO1) and target genes involved in plant defense against *B. cinerea* infection. Similar results were obtained also in tomato *Solanum lycopersicum* [56••]. By using stringent target prediction criteria, more than 70 Bc-siRNAs that are enriched during infection have predicted host targets in both *Arabidopsis* and tomato. It is worthwhile to investigate whether similar sRNA effectors that suppress host immunity also exist in other pathogens or pests.

B. cinerea possesses two DCL genes, both of which are required for the production of mobile Bc-sRNAs. Gene knockout of both *DCL*s in *B. cinerea* led to reduced virulence capacities due to the absence of plant immune-suppressing Bc-sRNAs [56••]. The majority of predicted Bc-sRNA effectors (including the three experimentally confirmed Bc-sRNAs: Bc-siR3.1, Bc-siR3.2 and Bc-siR5) are mapped to clusters within long-terminal repeat (LTR) retrotransposons in the genome of *B. cinerea*. Retrotransposons are hot spots of sRNA production for transposon silencing, a mechanism called quelling in fungi. Interestingly, these *Botrytis* LTR retrotransposons, called *Boty* elements, are genetically associated with virulence and host preference in natural populations of *B. cinerea* [58•], supporting the notion that these *Boty* elements give rise to sRNA effectors that enhance the pathogenicity of *B. cinerea*. Bc-sRNA effectors are physically linked to *Boty* elements and may facilitate fast turnover of Bc-sRNAs, which may provide an evolutionary advantage to pathogens in the arms race against host plants [4]. Similarly, fungal and oomycete protein effector genes are also enriched in the retrotransposon regions [59].

Taking advantage of environmental RNAi in invertebrates, scientists have engineered crop plants to express artificial sRNAs that can silence essential genes of plant–parasitic nematodes and herbivores (Figure 2b,c) [52,60–62]. Diverse host plant species have been successfully engineered to manipulate interacting pests in order to limit their virulence or to reduce their fecundity on host plants, to achieve advanced host resistance [51]. Furthermore, mobile silencing signals are not limited only to sRNAs. HIGS in the pest cotton bollworm was retained when they were fed on *dcl2dcl3dcl4* triple mutant *Arabidopsis* plants, suggesting that long dsRNA precursor rather than mature siRNAs are translocated, and which are likely to be processed in the bollworm to be functional [63]. This observation is consistent with long dsRNA uptake by insects and nematodes.

The animal–parasitic nematode *H. polygyrus* secretes vesicular miRNAs to suppress host immunity. It is well known that plant–parasitic nematodes feed on roots of plants that cause damages of the root system leading to reduced plant health and biomass production. Whether plant parasites also generate natural sRNA silencing signals to be translocated into host cells has yet to be explored. Thus, further research on the ability of pests and plant–parasitic nematodes to generate extracellular sRNAs that target plant immunity genes using host sRNA transport systems is of particular importance to the future of crop production (Figure 2b,c).

Mobile sRNAs or long dsRNAs, as cross-kingdom RNAi triggers, are fascinating; yet, it is enigmatic how these RNA molecules 'travel,' sometimes over long distances through diverse cellular boundaries between plants and interacting organisms. Cell-to-cell movement of plant sRNAs has previously been studied [8]. It is likely that mobile pathogen sRNAs can spread similarly from the site of infection into adjacent cells and impact the surrounding plant tissue. Importantly, sRNA transfer is not a random process through a concentration gradient, but rather a selective transport of functional sRNAs [64,65,66•,67]. This is supported by the fact that profiles of mobile sRNA pools are very different from the total sRNA populations within the cells. Such selective transport mechanisms could likely be overcome when the concentration of silencing signals reaches a high level, as in the case of HIGS. RNA-protective factors such as AGOs, other RNA-binding proteins, or encapsulation into extracellular vesicles likely play important roles in protecting mobile RNAs against degradation during transport [13•,14]. These RNA-binding proteins or transport machinery may also be involved in the sRNA selection process.

The parasitic plant dodder (*Cuscuta pentagona*) establishes a symplastic junction — via a haustorium — with their hosts to gain access to water and nutrients. Bidirectional transfer of thousands of mRNAs between *Cuscuta* and two hosts, *Arabidopsis* and tomato, has been observed [68•,69]. Host mRNA transcripts were tracked back in the dodder parenchyma at a distance of up to 30 cm away from the tomato/dodder connection [70]. Because the profiles of the transferred parasite mRNAs and the total mRNAs within the invaded cells are rather different, it is likely that selective transport is in action. However, the fate and function of these transferred mRNAs remains unclear, for example, whether these transferred transcripts are translated into functional proteins, or are degraded as a nutrient resource. The evidence of mRNA exchange makes it very likely that sRNAs that affect gene expression also travel bi-directionally via the haustorium (Figure 2d). This is supported by the successful application of HIGS against *Cuscuta* [71].

Mobile RNAs as ligands of Toll-Like Receptors in immune signaling

Circulating miRNAs have been shown to be internalized by recipient cells functioning as gene expression regulators.

Recent studies revealed that miRNAs can also act as ligands of Toll-Like Receptors (TLRs). TLRs are a conserved family of receptor proteins that play a major role in immune signaling in animals and plants. Two exosomal tumor-related miRNAs bind to murine TLR7 and human TLR8 in immune cells, activating a prometastatic inflammatory response [72•]. Interestingly, the mouse TLR13 recognizes a conserved 23S ribosomal RNA molecule of the bacterial pathogen *Staphylococcus aureus* and triggers an immune response [73••]. These findings suggest that conserved nucleic acids can serve in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). In the model plant *A. thaliana*, treatment of bacterial plasmid DNA was able to elicit PTI. DNA-induced PTI was abolished when plants were pre-treated with endocytosis-inhibitory chemicals suggesting that uptake of bacterial DNA was endocytosis-dependent [74]. Whether mobile sRNAs of plant-interacting pathogens/pests/parasites or symbionts act as signals to trigger a plant immune response, perhaps by binding to TLRs or other types of receptor-like proteins, needs to be investigated.

Biotechnological use of mobile sRNAs in plants

The discovery of sRNAs as mobile gene regulators creates exciting new opportunities to further investigate plant-pathogen interactions and to develop novel strategies for plant defense against pathogens and pests [50,51]. This is supported by the fact that HIGS has effectively worked in a variety of plant species against diverse plant herbivores, nematodes, and filamentous pathogens, when targeting important virulence genes. HIGS is also a well-established tool in specific host plant cultivars against particular pathogen strains under controlled lab-scale conditions. An important step remains to test the broader applicability of HIGS under field conditions, where HIGS plants are exposed to fluctuating environmental stresses that include pathogen and pest populations containing tremendous genetic variability, rather than clonal pathogens.

The fact that sRNA transport has been observed in plant–pathogen, plant–parasite, or plant–symbiotic interactions increases the possibility that beneficial fungi or disarmed pathogens (with essential virulence genes deleted) can be engineered to successfully manipulate plant physiology via *trans*-kingdom gene silencing (Figure 2e). Moreover, targeting pathogen mRNAs via harmless plant-interacting, organism-transmitted RNAi signals into associated plants has the potential to help defeat a broad range of pathogens and pests in a transgene-free plant framework. Thus, understanding the molecular mechanisms of RNA communications and transport between plants and interacting organisms will help improve RNA silencing-based technologies. While genetically modified crops remain a concern to some consumers, our advances in understanding cross-kingdom RNAi may help alleviate public concerns. Other applications of mobile sRNAs in plants are currently being discussed in regards to metabolic engineering and systemic-induced resistance [75,76]. Last but not least, food RNAi might become an important component of plant food-based technologies in the future [77]. Feeding studies revealed that oral uptake of sRNA-containing nutrients led to accumulation of food-borne sRNAs in body fluids and organs, indicating that high-dosage sRNAs can partially survive the intestinal track [78]. It is currently under investigation and debate whether food-borne sRNAs have any negative or positive impacts on the physiology of individuals who consume foods containing abundant sRNAs [79–82].

Conclusions

RNAs are considered to have cell-autonomous functions in gene expression and protein synthesis. Despite the fact that RNAs are vulnerable targets for nucleases, they are able to survive outside of a cell. Functional extracellular sRNAs move cell to cell and over long distances in plants, spread systemically in pests, and circulate via body fluids in mammals. Moreover, recent findings have demonstrated the unidirectional or bidirectional cellular exchange of sRNAs as silencing signals between hosts and pathogens/pests/parasites, or symbionts, in a phenomenon called cross-kingdom RNAi. Cross-kingdom RNAi influences host–pathogen interactions, for example, sRNAs from the plant pathogen *B. cinerea* and the mammalian parasite helminth nematode *H. polygyrus* translocate into host cells and suppress host immunity genes. On the other hand, however, plant-produced RNAi signals silence pathogen and pest genes, providing host resistance in a transgenic approach called HIGS. We speculate that additional pathogens also produce sRNAs for host immune suppression, while plants produce natural mobile sRNAs for defense by silencing genes in the interacting organisms.

Cell-to-cell transport mechanisms must exist for cross-kingdom RNAi. Secretion pathways and cellular uptake of RNAs have been described in animals. The nematode *C. elegans* has evolved unique RNA transporters (SID-1, SID-2) that are required for dsRNA uptake and systemic silencing. In mammals, functional miRNAs circulate through body fluids, often encapsulated in vesicles called exosomes. Release and uptake of vesicular sRNAs is mediated via endocytosis and exocytosis. Circulating miRNAs are probably protected against degradation by RNA-associated proteins, such as silencing proteins, AGO2 and GW182. We speculate that similar sRNA transport mechanisms, perhaps vesicle-based, also exist in plants and fungi. Remarkably, the helminth *H. polygyrus* secretes miRNAs in exosomal-like vesicles that are taken up by mammalian cells. Released nematode miRNAs target immune-related mRNAs and potentially suppress host immunity. We hypothesize that additional parasites and pathogens also hijack conserved RNA transport mechanisms existing in

their hosts to shuttle virulent sRNAs into host cells for immune suppression.

Several cases of cross-kingdom gene silencing have been observed, sometimes between interacting organisms that are phylogenetically unrelated, such as plants and fungi/insects/nematodes/symbionts, nematodes and bacteria, or mammals and parasites/nematodes. Some pathogen-secreted sRNAs mimic the endogenous sRNAs of their hosts, such as *Botrytis* sRNAs and *Heligmosomoides* miRNAs. Furthermore, RNA-mediated gene silencing is a ubiquitous phenomenon that exists in almost all eukaryotes, which always follows the principle of complementary nucleotide base pairing between regulatory sRNA and mRNA sequences. Despite the tremendous differences that are present in the structural features of regulatory RNAs (e.g. the differences between *E. coli* non-coding RNAs *OxyS* and *DsrA* and eukaryotic host siRNAs/miRNAs) and the completely unrelated or highly divergent RNA gene-silencing mechanisms and pathways that have evolved in diverse organisms, complementary sequence matches seem to be sufficient enough to trigger cross-kingdom gene-silencing. It seems that having intact RNAi machinery is not absolutely necessary. Cross-kingdom RNAi will be a valuable tool for future use in the development of novel therapeutic disease control and crop protection.

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