***Trans*-species mobility of RNA interference between plants and associated organisms**

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Abstract

Trans-species RNA interference occurs naturally when small RNAs (sRNA) silence genes in species different from their origin. This phenomenon has been observed between plants and various organisms including fungi, animals, and other plant species. Understanding the mechanisms used in natural cases of *trans*-species RNAi, such as sRNA processing and movement, will enable more effective development of crop protection methods using host-induced gene silencing (HIGS). Recent progress has been made in understanding the mechanisms of cell-to-cell and long-distance movement of sRNAs within individual plants. This increased understanding of endogenous plant sRNA movement may be translatable to *trans*-species sRNA movement. Here, we review diverse cases of natural *trans*-species RNAi focusing on current theories regarding intercellular and long-distance sRNA movement. We also touch on *trans*-species sRNA evolution, highlighting its research potential and its role in improving the efficacy of HIGS.

Introduction

Small non-coding RNAs (sRNAs) are critical in regulating numerous plant biological processes. One process in which they do so is RNA interference (RNAi), a gene silencing pathway that utilizes sRNAs to guide transcriptional gene silencing (TGS) and/or post-transcriptional gene silencing (PTGS). Within RNAi, sRNAs act as sequence-specificity determinants for RNA-induced silencing complexes (RISC) which initiate TGS or PTGS (Guo et al., 2016). Two major classes of sRNAs trigger RNAi in plants: microRNAs (miRNAs) and short interfering RNAs (siRNAs). Most genes that encode miRNAs (*MIRNA* genes) are transcribed by DNA-dependent RNA polymerase II to create primary transcripts that have an imperfect hairpin RNA secondary structure. This hairpin is recognized by a family of endonucleases called DICER-LIKE (DCL) proteins and, with the help of accessory proteins, is processed into a miRNA/miRNA* duplex (Hudzik et al., 2020). siRNAs are derived from double-stranded RNA precursors (dsRNA) that are often synthesized from an initial single-stranded RNA by an RNA-dependent RNA polymerase (RDR). siRNA precursors can be processed by one or more DCL proteins to create siRNA duplexes. siRNAs can be classified into subclasses according to their length, processing DCL proteins, and downstream function (Sanan-Mishra et al., 2021). siRNAs processed by DCL2 and DCL4 are 22 and 21 nucleotides, respectively, and are well characterized for their role in antiviral defense and PTGS. 24 nucleotide siRNAs primarily guide TGS via RNA-directed DNA methylation (RdDM) and are mostly processed by DCL3 (Jin et al., 2022).

Following DCL processing, one strand of a miRNA or siRNA duplex binds to an ARGONAUTE (AGO) protein and is incorporated into RISC. Subsequently, the AGO-bound sRNA guides RISC to the target region in a sequence-specific manner, initiating the silencing process. Plant AGO proteins primarily function as endonucleases. AGO endonuclease activity is crucial for initiating PTGS or TGS through the targeted cleavage of RNA molecules (Guo et al., 2016; Jin et al., 2022; Wang et al., 2023). In certain cases, when a mRNA is cleaved by a 22-nucleotide miRNA or siRNA, the resulting cleaved fragments can form dsRNAs, which are then processed by DCL proteins into functional sRNAs known as secondary siRNAs or phased siRNAs (Chen et al., 2010; Wu et al., 2020). The initiation of secondary siRNA biogenesis involves recruiting RDR6 to the 3' end of the cleaved transcript, which serves as a template for dsRNA synthesis. DCL2 and/or DCL4 process the newly synthesized dsRNA into secondary siRNAs, guiding the additional silencing of transcripts from which they originated. This production of secondary siRNAs by siRNAs- and miRNA-induced cleavage is referred to as transitivity and has been shown to promote the movement of siRNAs between cells and as a feed-forward loop for PTGS (de Felippes and Waterhouse, 2020).

Beyond its role in regulating essential plant functions, the RNAi pathway serves as an innate defense response against endogenous elements like transposable elements and exogenous RNAs from viral (Jin et al., 2022), parasitic nematode (Banerjee et al., 2017), and fungal pathogens (Zhang et al., 2016). This inter-species gene suppression has been termed *trans*-species RNAi or cross-kingdom RNAi. It is a naturally occurring process characterized by the transfer of small regulatory RNAs between different species. (We use the term *trans*-species RNAi because some cases of transfer between organisms are plant-to-plant, within the same kingdom). *Trans*-species sRNAs can function either in host defense or for the benefit of invading species, silencing genes associated with the immune response (Johnson et al., 2019), development (Zhang et al., 2022), or sRNA movement (Garnelo Gómez et al., 2021). This process plays an important role in disease and pathogen resistance in plants. RNAi presents an exciting alternative to pest and pathogen control in agriculture, with the potential to be both a sustainable and robust solution (Cai et al., 2018a).

There are several techniques for leveraging RNAi mechanisms to enhance plant resistance. In virus-induced gene silencing (VIGS), target gene fragments integrate into the host genome using viral vectors, triggering siRNA production. Successful VIGS requires intercellular and systemic spread of these virus-derived siRNAs (Rössner et al., 2022; Zulfiqar et al., 2023). However, in some instances, viral siRNAs spread ahead of full viruses, breaching meristem areas (Baulcombe, 2022; Bradamante et al., 2021; Incarbone et al., 2023). This primes antiviral defense, impeding VIGS in meristem tissues. Thus, understanding sRNA movement and tailoring strategies for each host tissue and target gene is crucial. Another technique is spray-induced gene silencing (SIGS). SIGS involves spraying or topically delivering dsRNAs onto the plants to silence pathogen or insect genes. The on-field application of SIGS faces

challenges due to the instability of naked dsRNA. Inspired by the role of extracellular vesicles (EVs) in natural *trans*-species RNA transport seen in *Botrytis cinerea* and *Arabidopsis* (Cai et al., 2018b; He et al., 2021; Wang et al., 2016), artificial nanovesicles resembling EVs have been used to improve RNA stability and internalization in SIGS (Chen et al., 2023; Qiao et al., 2023). Collectively, understanding natural intercellular and *trans*-species gene silencing can help improve existing methods and develop new tools in artificial RNAi. Host-induced gene silencing (HIGS), introduced by (Nowara et al., 2010), utilizes a transgenic host continuously producing and delivering dsRNAs, hairpin RNA, or sRNAs to pests or pathogens, specifically targeting virulence genes (Koch and Wassenegger, 2021). HIGS has demonstrated effectiveness in various organisms, including the parasitic plant *Cuscuta pentagona* (Alakonya et al., 2012). When cultivated on transgenic tobacco expressing hairpin RNA against the *Shoot Meristemless-like* (*STM*) gene, *C. pentagona* exhibited reduced STM expression and poor growth (Alakonya et al., 2012). This successful application of HIGS laid the foundation for the subsequent discovery of the natural exchange of sRNAs between plants and *Cuscuta* species (Shahid et al., 2018; Johnson et al., 2019). A recent method for conferring artificial RNAi is microbe-induced gene silencing (MIGS) (Wen et al., 2023). In MIGS, a modified beneficial fungus induced gene silencing in nearby pathogenic fungi, protecting cotton and rice plants (Wen et al., 2023). The success of MIGS reveals the possibility of natural *trans*-species RNAi between rhizospheric microorganisms. Advances in artificial RNAi, including HIGS, SIGS, VIGS, and MIGS, unveil more naturally occurring *trans*-species cases. These advancements underscore the versatile use of RNAi to manipulate gene expression not only within an organism but also across different species. The knowledge about how gene silencing naturally occurs across species provides a foundation for developing innovative tools in artificial RNAi. This could involve designing more targeted and efficient delivery systems, optimizing the stability of RNA molecules, and exploring novel strategies to enhance the specificity and potency of gene silencing.

In this minireview, we highlight recent studies of the roles of mobile sRNAs within the organism and *trans*-species scale. We delve into key mechanisms of naturally occurring *trans*-species RNAi with a particular focus on how movement across the species barrier may occur. We also talked about various evolutionary strategies employed by organisms to target multiple transcripts across diverse host species, even in the face of selective natural pressures.

***Trans*-species RNAi natural mechanisms**

In recent decades, *trans*-species RNAi has become a useful tool for developing crop varieties with enhanced resistance to a range of pests and pathogens, as well as for conducting functional and metabolic studies. One technique often leveraged for this purpose, HIGS, involves engineering hosts to produce sRNAs or sRNA precursors designed to target pathogen/parasite mRNAs. Successful application of HIGS (as well as other RNAi techniques) against pests requires a deep understanding of pest and host interactions. This includes characterization of interspecies effectors, delivery methods, and the movement of sRNAs between organisms. There are several examples of naturally occurring *trans*-species RNAi that have been described from diverse plant-pest and plant-pathogen interactions. These natural examples may serve to inform more effective HIGS strategies.

Fungi

Since its discovery, *trans*-species RNAi has been a focus for developing fungal-resistant crops. For example, *Verticillium dahliae*, a pathogenic fungus responsible for wilt disease in various crops, poses a significant agricultural threat, necessitating a sustainable and robust control method (Dubrovina et al., 2019). In *V. dahliae*, naturally occurring *trans*-species RNAi has been observed. A 24 nucleotide *V. dahliae*-derived sRNA (Vd-sRNA) was found to target *Arabidopsis* miR157d, a critical regulator of *Squamosa-Promoter Binding Like* (*SPL*) genes (Zhang et al., 2022). Expression levels of two *SPL* genes (*SPL13A/B*) were reduced in hosts as a result of *V. dahliae* infection, leading to delayed floral transition for *Arabidopsis*, presumably to aid in further infection (Zhang et al., 2022). Vd-sRNAs between 20 and 24 nucleotides immunoprecipitated with host AGO1, suggesting that *V. dahliae* sRNAs associate with host RNAi machinery to silence host transcripts (Zhang et al., 2022). These results provided evidence of *trans*-species RNAi between a fungal species and host plant, along with the resulting phenotypic effects on the host.

Plants have been shown to transfer sRNAs to fungal pathogens for RNAi defense as well. Cotton plants infected with *V. dahliae* expressed miR159 and miR166 at higher levels than uninfected plants (Zhang et al., 2016). These miRNAs were determined to target two *V. dahliae* mRNAs, *Ca^{2+} dependent cysteine protease calpain* (*Clp-1)* and *isotrichodermin C-15 hydroxylase* (*HiC-15)*. *Clp-1* is required for alkaline stress tolerance in fungi, while *HiC-15* is involved in the production of trichothecene metabolites (Zhang et al., 2016). These genes were independently knocked out in *V. dahliae,* with unique phenotypes upon colonization. However, while neither exhibited significant loss in biomass, both knockout mutants had reduced virulence in cotton plants and no longer caused wilt symptoms. These results provide evidence of host-derived regulation of fungal virulence (Zhang et al., 2016).

Trans-species RNAi was found to play a crucial role in the pathogenicity of another necrotrophic fungal species, *Botrytis cinerea,* on hosts *Arabidopsis* and tomato. Upon inoculation, the fungus exports a myriad of sRNAs into the hosts (Weiberg et al., 2013) (**Fig. 1A**). These *B. cinerea* sRNAs (Bc-sRNAs) were shown to functionally silence important defense-related genes in both hosts including *Mitogen-Activated Protein Kinases* (*MAPK*), which regulate jasmonic acid and ethylene levels for plant defense (Weiberg et al., 2013). Many of the Bc-sRNAs were 20 to 22 nucleotides long, with 5' terminal U, making them quite similar to plant miRNAs. Results suggest Bc-sRNAs are processed by *B. cinerea* DCL1 and DCL2 (Bc-DCL1 and Bc-DCL2), then upon exportation into hosts, are bound to host AGO1 to silence host genes (Weiberg et al., 2013) (**Fig. 1A**). The movement of sRNAs between *B. cinerea* and host plants is bi-directional (Cai et al., 2018b) (**Fig. 1A**). In response to the infection*, A. thaliana* sends defensive sRNAs into *B. cinerea* (Cai et al., 2018b) (**Fig. 1A**). When impairing the biogenesis of these host-derived sRNAs by mutations in *DCL* or *RDR* genes, *A. thaliana* had higher susceptibility to *B. cinerea* (Cai et al., 2018b). This suggests that *A. thaliana* delivers host sRNAs into fungal cells to silence virulence-related genes. The two-way motion of *trans*-species sRNAs is further illustrated through artificial RNAi. Hairpin RNAs targeting *Bc-DCL1* and *Bc-DCL2* were stably expressed in transgenic *A. thaliana* and transiently expressed in tomato plants. Following infection in these transgenic hosts, there was a significant reduction in the expression of *Bc-DCL1* and *Bc-DCL2*. This indicates that artificial hairpin RNA or its derivative sRNAs traversed from the host to the parasite, leading to the silencing of specific *B. cinerea* targets (Wang et al., 2016).

In addition to defensive and pathogenic functions, *trans*-species RNAi has been proposed as a beneficial mechanism between symbiotic microorganisms and plants, such as with *Rhizophagus irregularis*, an arbuscular mycorrhizal fungus, and its host *Medicago truncatula* (Silvestri et al., 2019). Populations of sRNAs in *R. irregularis* were characterized and several fungal sRNAs were predicted to target *M. truncatula* transcripts (Silvestri et al., 2019). Some of these putative target genes, such as *Developmentally Regulated Plasma Membrane Polypeptide* (*MtDREPP*) and *Responsive to Dehydration 22* (*RD22*), were shown to be downregulated in host roots colonized with mycorrhizal compared to non-mycorrhizal roots (Silvestri et al., 2019). MtDREPP is thought to play a role in plasma membrane remodeling, and its modulation may be required for the colonization of arbuscular mycorrhizal fungus. RD22 is an abscisic acid-responsive gene involved in pathogen susceptibility, which may be regulated to promote *R. irregularis* colonization (Silvestri et al., 2019). The finding suggests that *trans*-species RNAi occurs not only as a pathogenic mechanism, but also in mutualistic symbiosis. Indeed, further support of *trans*-species RNAi mechanisms between a beneficial fungal species and plant was seen in *Fusarium solani*. Artificial RNAi in *F. solani* strain K (FsK), a beneficial fungus, was found to be capable of silencing and directing DNA methylation of a host reporter gene (GFP) in *Nicotiana benthamiana* (Dalakouras et al., 2023) Although this case is not a naturally occurring instance of RNAi, it does support a mechanism for *trans*-species RNAi between beneficial fungi and host plants.

These studies provide evidence of *trans*-species sRNAs function, but one consideration is how these *trans*-species RNAs can move between fungi and host plants. Within fungi, sRNAs can move through septal pores (a plasmodesmata-like structure) or extracellular vesicles (EVs), allowing intercellular transport (Wang and Dean, 2020). It has been suggested that *trans*-species RNA movement between fungal pathogens and host plants is carried out by deployment of vesicle-contained sRNAs (Wang and Dean, 2020). The transport of RNAs via extracellular EVs is a well-characterized process in fungal species (Kwon et al., 2020). However, the involvement of EVs in *trans*-species sRNA exchange between plants and fungal pathogens remains an active area of research. Recent studies, such as the one

conducted by (He et al., 2021) investigated EVs within *Arabidopsis* and the role of RNA-binding proteins (RBPs) in sRNA movement. The study found that certain RBPs such as AGO1 and RNA helicases (RH11, and RH37) were selectively bound to EV-enriched sRNAs in *Arabidopsis* (**Fig. 1A**). Additional evidence indicated that hosts deficient in these RBPs were not only more susceptible to *B. cinerea* infection, but the fungal target genes were no longer suppressed. This supports the notion that sRNAs can be transferred from plants to fungi through EVs, and likely involve RBP binding (**Fig. 1A**).

Animals

HIGS has been successful against diverse organisms within the animal kingdom, including various pathogenic nematode species (Zhuo et al., 2017; Blyuss et al., 2019; Iqbal et al., 2021) and insects (Eakteiman et al., 2018; Fishilevich et al., 2019; Sun et al., 2019). Successful HIGS experiments suggest that sRNA precursors are likely taken up by parasitic nematodes and other animals when consuming host material containing double-stranded RNA (Dutta et al., 2015). One species of parasitic nematode of interest is *Meloidogyne incognita,* a parasitic root-knot nematode that causes extensive damage to host plants including wilting, stunted growth, and impaired immunity to disease (Blyuss et al., 2019). *M. incognita* lays eggs on the roots of host plants, which hatch and burrow into host roots. This process triggers gall formation and the feeding of host material. There have been several attempts at using engineered RNAi methods against *M. incognita* with varied success (Dutta et al., 2015; Hada et al., 2021; Iqbal et al., 2021), which suggests delivery of sRNA or sRNA precursors to the parasite is possible. These results show that while some parasite genes are recalcitrant to RNAi for unknown reasons, others are more susceptible to knockdown and have a significant impact on parasite development (Iqbal et al., 2021). This includes PTGS of parasite RNAi machinery such as *M. incognita* DICER (*Midcr-1.1)*. Iqbal et al. (2021) tested several dsRNA constructs that targeted regions of *Midcr-1.1,* resulting in a successful knockdown, impaired pathogenicity of *M. incognita,* and evidence of RNAi susceptibility. One instance of naturally occurring *trans*-species RNAi was observed in a honeybee-plant interaction (Zhu et al., 2017). It was observed that bee bread produced from pollen, a diet eaten by worker bee larvae, contained sRNAs derived from *Brassica campestris*. These sRNAs were determined to have targets within honeybees. One miRNA in particular, miR162a, was found to target the *Target Of Rapamycin* (*amTOR*), a gene responsible for caste development in bees (Zhu et al., 2017). The implications of developmental RNAi in honeybees derived from pollen-based food sources still require further investigation, but these results suggest a potential naturally occurring gene regulatory mechanism from dietary sources in honeybees.

Plants

Plants have several documented cases of exogenous sRNA transfer within and between species. *Arabidopsis* has been reported to take up and use exogenously supplied miRNAs, triggering gene silencing in the recipient plant through hydroponic solutions (Betti et al., 2021). This suggests that *A. thaliana* is capable of both releasing and absorbing sRNAs from its environment, leading to intra-species gene silencing. Additionally, plant-to-plant sRNA-mediated silencing has been observed to transcend species boundaries. Heterografting experiments showed that transgene-derived sRNAs moved from potato rootstock to suppress target genes in tobacco scion (Kasai et al., 2016). Other studies have documented the exchange of endogenous sRNAs between various species of grapevines and sweet cherry trees (Zhao et al., 2020; Rubio et al., 2022). These collective findings underscore the remarkable capacity of sRNAs, whether originating internally or externally, to move between distinct species, likely facilitated by continuous vascular connections.

The *trans*-species movement of sRNAs is illustrated in parasitic plants through HIGS. Parasitic plants connect with hosts through vascular links, akin to “natural grafting.” In HIGS, transgenic host plants express siRNAs, which enter parasites to silence corresponding virulence genes and boost host plant tolerance. For example, in the case of the obligate parasitic plant *Cuscuta*, host-derived sRNAs targeting genes involved in haustorium organogenesis impede parasite growth (Alakonya et al., 2012; Jhu et al., 2022, 2021). The success of HIGS approaches in *Cuscuta* hinted at the potential for the natural exchange of sRNAs between *Cuscuta* and host species, later proven true. When parasitizing *Arabidopsis* and tobacco, *Cuscuta* synthesizes novel miRNAs, mostly 21 and 22 nucleotides in length, at the haustorial interface (Shahid et al., 2018; Johnson et al., 2019; Hudzik et al., 2023) (**Fig. 1B**). These miRNAs, termed interface-Induced miRNAs (IIMs), began to accumulate two days after *Cuscuta* has successfully wrapped around a host and can be consistently detected until day 14 (**Fig. 1B**). These findings indicate that

Cuscuta-derived IIMs emerge in the early stages of haustorium development, preceding any penetration of the host tissue. Several *Cuscuta* IIMs have been confirmed to exhibit *trans*-species activity by targeting host mRNAs (Shahid et al., 2018). They can utilize the host plants' silencing machinery to initiate the generation of secondary siRNAs. The incoming miRNAs, perhaps with assistance from secondary siRNAs, lead to the degradation of host mRNAs (Shahid et al., 2018) (**Fig. 1B**). In a study conducted by (Subhankar et al., 2021), it was revealed that sRNAs originating from *C. campestris* could traverse considerable distances within recipient plants, including reaching distal organs such as the apical meristem. However, the specific features that distinguish these interface-induced miRNAs from canonical miRNAs and enable them to undergo *trans*-species movement are still under investigation. In the study by (Hudzik et al., 2023), *Cuscuta* IIM genes were found to possess U6-like small nuclear RNA (snRNA) promoters with a characteristic upstream sequence element (USE) (**Fig. 1B**). Notably, the primary transcript of these IIMs suggests their synthesis by RNA polymerase III, distinguishing them from other canonical sRNAs in plants (Hudzik et al., 2023) (**Fig. 1B**). These distinctive features may enhance their export to host plants, and potentially shed light on the long-standing mystery of *trans*-species sRNAs export between plants.

Movement of sRNAs in plants and associated organisms

Strategies that suppress genes required for *trans*-species sRNA biogenesis, such as DCL proteins, have proven effective in protecting host plants (Wang et al., 2016; Werner et al., 2020). Alternatively, a potential strategy for safeguarding host plants involves disrupting sRNA transmission from parasites to hosts. Yet, our understanding of how sRNA-mediated silencing spreads across species remains limited. Insights from the transport mechanisms of within-organism plant sRNAs may offer valuable information about *trans*-species sRNA movement. Short-distance, cell-to-cell movement of canonical sRNAs likely occurs via plasmodesmata (Voinnet et al., 1998; Long et al., 2021; Schröder et al., 2023). Evidence supporting this is the absence of silencing occurred in the symplastically isolated stomatal guard cells after the agroinfiltration (Voinnet et al., 1998). However, once the silencing signal had reached the apical region, the guard cells on the newly formed leaf were steadily silenced (Voinnet et al., 1998). This is because the signal enters the leaf before guard cells close plasmodesmata with the remainder of the leaf cells (Voinnet et al., 1998). A prevailing hypothesis for long-distance movement of regular sRNAs involves production within source cells, followed by diffusion into companion cells and subsequently into sieve tube elements, rendering them phloem-mobile (Ham and Lucas, 2017, 2014; Subramanian, 2019; Yan, 2022). These phloem-mobile sRNAs are released from the phloem at sink tissues and diffuse between cells to reach their destination.

The exact nature of the mobile entities in plant RNAi has long been discussed. The mobile molecules could theoretically be single-stranded mature sRNAs, duplexes, single- or double-stranded precursors, AGO-bound sRNAs, or some combination thereof. An *Arabidopsis dcl2/dcl3/dcl4* triple mutant where most siRNAs are absent was grafted with wild-type plants expressing a GFP-derived hairpin RNA (Molnar et al., 2010). GFP-specific siRNAs from the scion were detected in the triple mutant root (Molnar et al., 2010). This demonstrated that precursor movement cannot entirely explain the long-distance appearance of siRNAs. Further support for the mobility of siRNAs, rather than their precursors, was found in another grafting experiment (Brioudes et al., 2021). siRNA duplexes are stabilized through 2’-O-methylation of the 3’-most nucleotide. This methylation is catalyzed by Hua Enhancer 1 (HEN1) in the nucleus. To enable siRNA precursors to functionally transmit through grafting, they would require HEN1 activity in the recipient cells. However, following grafting, the levels of processed siRNAs were equal in *hen1* rootstocks in comparison to wild-type rootstocks (Brioudes et al., 2021). This is inconsistent with the notion of mobile siRNA precursors. This inconsistency prompts a reconsideration of the mobile agent's identity. One model is that the mobile agent is not a siRNA precursor, thus narrowing possibilities to non-AGO-loaded siRNA (single-stranded or duplexes) or AGO-bound siRNA. The viral silencing suppressor P19 exclusively binds to sRNA duplexes of 21 or 22 nucleotide (Skopelitis et al., 2018; Garnelo Gómez et al., 2021). Through P19 immunoprecipitation, siRNA duplexes sourced from phloem were recovered in root epidermal cells (Devers et al., 2020). This suggests that sRNA duplexes are mobile. Additionally, following the transient expression of artificial miRNA constructs, the miRNA:miRNA* ratio approximates one in non-infiltrated upper leaves that experience mobile miRNA activity (Cisneros et al., 2022). These findings underscore the mobility of siRNA and miRNA duplexes.

While existing evidence suggests that sRNA duplexes are likely the mobile agents within-organism, the nature of *trans*-species sRNA is still under investigation. The pathogenicity of *Botrytis cinerea dcl1dcl2* double mutant is diminished, and the mutant also lost the ability to produce *trans*-species Bc-sRNAs (Wang et al., 2016; Weiberg et al., 2013). The result suggests that the dicing of the Bc-sRNAs precursor occurs in the pathogen, and either the duplex or single-stranded sRNA is subsequently transported into the host. Similarly, when disrupting RNA biogenesis, *dcl2/3/4 Arabidopsis* is incapable of producing *trans*-species sRNA to inhibit *B. cinerea* virulence (Cai et al., 2018b). This, again, reaffirms that it is the incipient organism that completes the dicing of *trans*-species sRNAs. The evidence of sRNA duplexes or single-stranded sRNAs being the form sent into the recipient organism is also demonstrated in *Cuscuta campestris*. The detection of *trans*-species miRNAs in artificial haustoria of *C. campestris* serves as direct evidence that the processing of *trans*-species miRNA precursors takes place within *C. campestris* cells (Hudzik et al., 2023) (**Fig. 1B**). Consequently, it is plausible that the exported molecule could be either the mature miRNA or the miRNA/miRNA* duplex. This potential export might involve novel interactors, such as RNA binding protein and extracellular vesicles in the *B. cinerea* case, that play a role in safeguarding and facilitating transportation to host tissues.

It has been shown that AGO1, the major plant AGO for miRNAs and 21 nucleotide siRNAs, is cell-autonomous (Brosnan et al., 2019; Fan et al., 2022). Thus it is unlikely that AGO-bound sRNAs are the mobile version of the silencing agent in cell-to-cell movement within a plant. In fact, AGO loading has been demonstrated to limit the extracellular movement of siRNA duplexes (Devers et al., 2020). The majority of phloem-derived siRNAs that reached the root epidermis after grafting lacked 5' U, a characteristic feature of the AGO1 association (Mi et al., 2008). This observation suggests that AGO1 is the gatekeeper limiting the travel distance of these extracellular siRNAs by progressively sieving them out (Voinnet, 2022; Devers et al., 2023) (**Fig. 1C**). In the case of sRNAs serving as non-cell autonomous mobile signals, there might be a mechanism facilitating their production at a rate exceeding their consumption by AGOs, thus the surplus sRNAs can travel extracellularly. Consistent with this idea, miR165 and miR166 require KATANIN1 (KTN1), a microtubule-severing enzyme component for movement (Fan et al., 2022). KTN1 functions within endodermis cells to inhibit miR165/6 loading onto cytoplasmic AGO1. This inhibition promotes the cell-to-cell movement of miR165/6 into protoxylem and creates a gradient expression of their target *PHABULOSA*. Consequently, this gradient expression patterns the xylem cell fate in *Arabidopsis* roots (Fan et al., 2022) (**Fig. 1D**). Moreover, KTN1's role extends to the movement of exogenous miRNAs. Disrupting KTN1 limited the artificial miRNA (amiR)-mediated silencing of *SULFUR*, a key gene in chlorophyll synthesis, resulting in diminished leaf chlorosis (**Fig. 1D**). KTN1 also impacts the long-distance movement of artificial miRNA. In micrografts with *ktn1* rootstock and amiR scion, a significant amount of amiR-SUL was observed, indicating successful movement from shoot to root (**Fig. 1D**). Conversely, when *ktn1* served as a scion, the amiR-SUL level was markedly lower (**Fig. 1D**). These findings highlight the crucial role of microtubules in source tissues for the movement of sRNAs, both within cells and over long distances. This remains consistent regardless of the origin of the sRNAs, whether endogenous or exogenous, primarily by impeding their loading into cytoplasmic AGO1 (Fan et al., 2022). A preprint (Herridge et al., 2023) showed that specific plant siRNAs and miRNAs have significant amounts of pseudouridine (Ψ). Ψ is an isomer of U that is a common post-transcriptional modification of RNAs, perhaps most prominently appearing in the TΨC loop of transfer RNAs. (Herridge et al., 2023) reported that Ψ-enriched miRNAs and siRNAs are more likely to be mobile in both plants and animals. The mechanism by which Ψ enhances sRNA movement is not known. One possibility is that heavy Ψ modification of sRNAs somehow reduces AGO binding, thus allowing for movement. The simplest hypothesis is that the same rules of sRNA mobility apply to the movement of sRNA between pests and plants. Therefore, future investigations that examine avoidance of AGO-loading in source organisms and the presence of Ψ in *trans*-species sRNAs may be fruitful.

Trans-species sRNAs likely face demanding biological circumstances as they navigate between two organisms with differing internal conditions. Moreover, they confront potential perils such as exposure to RNases, phagocytosis, and extreme pH levels during their voyage. To prevent degradation, extracellular RNAs may either form close connections with RNA-binding proteins (RBPs) or become enclosed within extracellular vesicles (EVs). EVs shield their cargo from breakdown by external enzymes, a critical safeguard for RNA transport. Out of the 42 plant sRNAs transferred to the fungal pathogen *Botrytis*

cinerea, 31 were detected in EVs (Cai et al., 2018b). This observation indicates a potential role for EVs in facilitating the transport of sRNAs from plant cells to fungal pathogens.

Conversely, fungal pathogens also encase their sRNAs within EVs, which are then internalized by plant cells through clathrin-mediated endocytosis (He et al., 2023) (**Fig. 1A**). This highlights the potential of EVs in facilitating the bidirectional transport of sRNAs between distinct species. Notably, when facing *B. cinerea* infection, the host prioritizes the transfer of a particular set of sRNAs (Cai et al., 2018b). This selectivity is evident as the expression profiles of EV-enriched sRNAs and total sRNAs from the same tissue differ significantly (Cai et al., 2018b). Furthermore, the size distribution of sRNAs differs from those found in isolated EVs and apoplastic fluid (Baldrich et al., 2019). Taken together, these discoveries imply that the movement of *trans*-species sRNAs isn't exclusively propelled by passive diffusion driven by concentration gradients. Instead, it potentially entails a more precise mechanism that selectively loads *trans*-species sRNAs into EVs (Cai et al., 2021; He et al., 2021).

RBPs are central for loading and stabilizing *trans*-species sRNAs in EVs. Specific RBPs, like AGO1 and RNA helicases (RH11 and RH37), bind exclusively to EV-sRNAs, and Annexins 1 (ANN1) and ANN2 enhance sRNA stability within EVs (He et al., 2021) **(Fig. 1A)**. Disrupting these RBPs significantly reduces sRNA secretion into EVs, suggesting their importance in sorting and stabilizing *trans*-species sRNAs (He et al., 2021). This finding highlights the versatility of AGO1, which is considered cell-autonomous within a single organism, revealing its capacity to travel as EV cargo at the *trans*-species level. However, debate persists regarding the location of the sRNA-RBP complexes. Some studies (Cai et al., 2018b; He et al., 2021) indicate the presence of these complexes within EVs, while others (Baldrich et al., 2019; Zand Karimi et al., 2022) suggest that sRNA-RBP complexes exist outside EVs or closely associated with EV outer surface. Additionally, some EVs contain tiny RNAs ranging from 10 to 17 nucleotides, adding another layer of complexity to the scenario (Baldrich et al., 2019). To date, there is no evidence that tiny RNAs are components of RISC complexes. The differences in these findings are likely influenced by various factors, including the plant's specific growth conditions and methodological differences for EV purification. The profile of sRNA secreted during the healthy and infected stages can be disparate. For instance, *C. campestris* produces *trans*-species miRNA exclusively during parasitism; the miRNAs do not accumulate outside of tissues specialized for host contact (Shahid et al., 2018; Hudzik et al., 2023). The result of (Cai et al., 2018b; He et al., 2021) is based on infected hosts, while (Baldrich et al., 2019; Zand Karimi et al., 2022) used healthy plants. Further experiments treating infected host EVs with protease plus RNase may pinpoint the exact location of *trans*-species sRNA-RBP complexes. Several studies clarified that *trans*-species sRNAs might be associated with RBPs, traveling within, adjacent to, or outside of EVs (Cai et al., 2018b; Baldrich et al., 2019; He et al., 2021; Zand Karimi et al., 2022). Upon reaching the recipient organism, the mechanism by which *trans*-species sRNAs disassociate from RBPs and re-bind with AGO1 presents an intriguing challenge (**Fig. 1A**).

Evolution of *trans*-species sRNAs

Several instances of *trans*-species sRNAs in plants are believed to benefit only one of the interacting organisms (the pathogen or parasite). Presumably, the recipient organism would benefit from the avoidance of targeting in most cases. This raises the question of whether, and how, *trans*-species sRNAs maintain sequence complementarity to recipient organism mRNAs. Two paradigms have been described: the “shotgun” strategy, where incipient organisms produce an assorted set of sRNAs that target recipient organisms randomly. For instance, most *trans*-species siRNAs in *B. cinerea* originate from the retrotransposons (Porquier et al., 2021). These transposons exhibit significant sequence variability, offering an advantageous landscape for targeting various mRNAs across different host species. Similarly, when infected by the oomycete *Phytophthora, Arabidopsis* produces a diverse pool of secondary siRNAs to target multiple *Phytophthora* transcripts (Hou et al., 2019). Inhibition of this secondary siRNA production, as seen in *rdr6* and *sgs3* mutants, heightens host susceptibility, leading to severe disease symptoms (Hou et al., 2019). Both examples exemplify the 'shotgun' strategy, enhancing the likelihood of targeting multiple transcripts in diverse host species.

Conversely, a different paradigm is present in *Cuscuta*, where miRNAs are strategically employed to target host genes. Unlike siRNAs and secondary siRNAs, miRNAs are precisely excised from their precursors, yielding a singular functional product. *Cuscuta*-derived *trans*-species miRNAs target highly

conserved regions in host mRNAs. *Cuscuta* miRNAs also possess polymorphic sites that correspond precisely to synonymous sites in host target mRNAs. The combination of targeting highly conserved sites with synonymous-site polymorphisms likely prevents the host mRNAs from escaping the parasite miRNAs (Johnson et al., 2019; Hudzik et al., 2020). In both paradigms, sequence variations in host targets, aimed at evading silencing, are met with corresponding adjustments in the pathogen's sRNA sequences. This raises a question: Do these two paradigms extend to all instances of natural *trans*-species RNAi? To address this, studying more cases of *trans*-species sRNA-mediated silencing will be crucial. For instance, there are about 4,500 species of parasitic plants and the parasitic plant lifestyle has independently arisen multiple times (Nickrent, 2020; Ibiapino et al., 2022; Zangishei et al., 2022). Many thousands of diverse plant pathogens are also known. Mutualistic interactions, as observed in ectomycorrhizal fungi and host plants, also involve *trans*-species sRNAs (Wong-Bajracharya et al., 2022). Mutual relationships can turn neutral or parasitic under different conditions (Nakazawa and Katayama, 2020; Drew et al., 2021; Harrower and Gilbert, 2021), making it intriguing to investigate sRNA profiles during such shifts. Future work directed at systematically examining natural cases of *trans*-species RNAi will enhance the understanding of how RNAi can move between plants and their associated organisms. The study of *trans*-species sRNAs would involve extending the research to organisms that have evolved across varying timeframes, adopted different lifestyles, and engaged in diverse symbiotic interactions.

Conclusions and Perspective

Trans-species RNAi is a natural phenomenon observed in a variety of plant-pathogen, plant-parasite, and plant-symbiote interactions that has expanded the understanding of sRNA function in plants. The elucidation of mechanisms governing the biogenesis, transport, and subsequent silencing mediated by *trans*-species sRNAs has paved the way for the development of effective *trans*-species RNAi methods in plants. This includes introducing new potential methods of pest and pathogen control. One of the key unanswered questions is the mechanism of sRNA movement within and between species. Evidence within plants suggests that extracellular movement is directed by phloem-mobile sRNA duplexes (Devers et al., 2020; Cisneros et al., 2022). It is hypothesized that two mechanisms may be at work to limit transfer via the sieve tube elements. AGO1 may act as a gatekeeper, limiting which sRNAs are mobile between plant cells by inhibiting travel distance (Voinnet, 2022; Fan et al., 2022; Devers et al., 2023). Alternative hypotheses propose that modifications to sRNAs might contribute to their mobility. Ψ-enriched sRNAs exhibit enhanced mobility in plants and animals (Herridge et al., 2023). Specific RNAi machinery and transport mechanisms involved in *trans*-species RNAi require a careful evaluation of individual interactions between plants and interacting organisms. For instance, debates persist regarding the role of EVs and RBPs in the selective transport of sRNAs within and between plants and fungi (Baldrich et al., 2019; He et al., 2021). Another intriguing avenue still to be explored is the evolution of *trans*-species sRNAs. *B. cinerea* and *Cuscuta spp.* illustrate two distinct strategies for stabilizing sRNA-target relationships: either by enhancing sequence variability in the produced sRNAs or by targeting the highly conserved regions of the target. Exploring the evolution of *trans*-species RNAs in various organisms could provide insight into how different species employ *trans*-species RNAi over time. While more research is necessary to delve into the specific mechanisms and functionalities of *trans*-species sRNAs, the current progress lays the foundational cornerstone for understanding sRNA-mediated silencing across organisms from diverse species and kingdoms.

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Author Contributions

AZ and YN contributed equally to the research and writing of this manuscript. YN prepared the figure. MJA edited and provided overall guidance to the writing.

Disclosures

The authors declare no conflict of interest.

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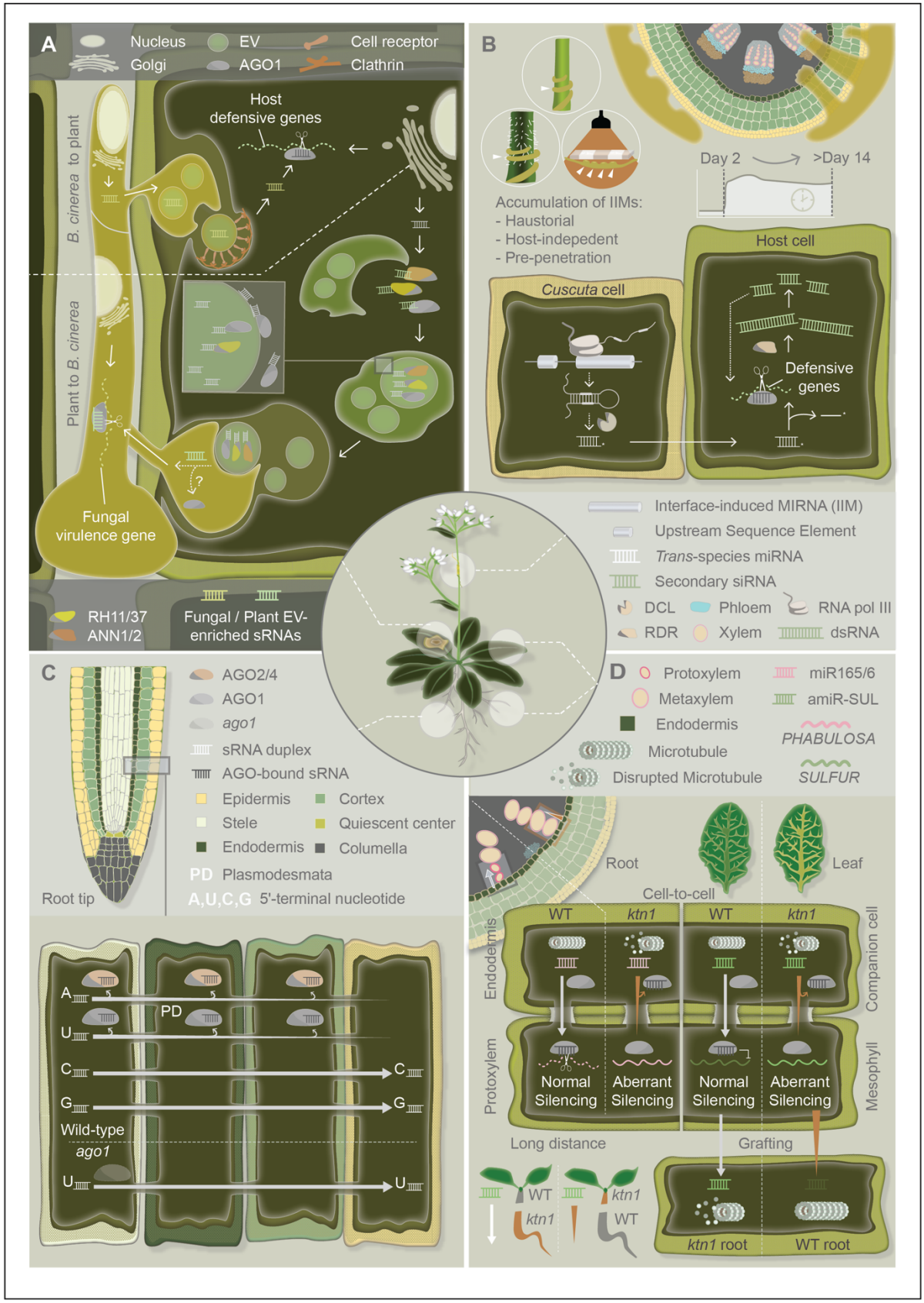
A
Nucleus
Golgi
EV
AGO1
Cell receptor
Clathrin
Host defensive genes
B. cinerea to plant
Plant to B. cinerea
Fungal virulence gene
RH11/37
ANN1/2
Fungal / Plant EV-enriched sRNAs
B
Day 2
>Day 14
Accumulation of IIMs:
- Haustorial
- Host-indepedent
- Pre-penetration
Cuscuta cell
Host cell
Defensive genes
Interface-induced MIRNA (IIM)
Upstream Sequence Element
Trans-species miRNA
Secondary siRNA
DCL
Phloem
RNA pol III
RDR
Xylem
dsRNA
C
AGO2/4
AGO1
ago1
sRNA duplex
AGO-bound sRNA
Epidermis
Cortex
Stele
Quiescent center
Endodermis
Columella
PD Plasmodesmata
A,U,C,G 5'-terminal nucleotide
Root tip
D
Protoxylem
Metaxylem
Endodermis
Microtubule
Disrupted Microtubule
miR165/6
amiR-SUL
PHABULOSA
SULFUR
Root
Leaf
Cell-to-cell
WT
ktn1
Endodermis
Protoxylem
Companion cell
Mesophyll
Normal Silencing
Aberrant Silencing
Long distance
Grafting
ktn1 root
WT root
A
U
C
G
PD
Wild-type
ago1

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Figure Legends

Figure 1. Summary of key findings related to *trans*-species (A and B) and intercellular/within species (C and D) movement of sRNA-mediated silencing. (A) Bidirectional *trans*-species sRNA between *Botrytis cinerea* and host plants (Cai et al., 2018b; He et al., 2021; He et al., 2023). EV: Extracellular vesicle, AGO1: ARGONAUTE 1, RH: RNA helicases, ANN: ANNEXINS. Upon reaching the recipient organism, the mechanism by which *trans*-species sRNAs disassociate from RNA-binding proteins and re-bind with AGO1 is unknown ('?'). **(B)** Expression profile, biogenesis, and function of *C. campestris*-derived interface-induced miRNAs (IIMs) (Shahid et al., 2018; Johnson et al., 2019; Hudzik et al., 2023). White arrows indicate haustoria, DCL: DICER-LIKE protein, RDR: RNA-dependent RNA polymerase, RNA Pol III: RNA polymerase III, dsRNA: double-stranded RNA. **(C)** Consumption of sRNAs by AGO1 limits sRNA movement in the root tip, featuring a 5' terminal nucleotide discrepancy (Voinnet, 2022; Devers et al., 2023). *ago1*: ARGOUNAUTE 1 mutant. **(D)** Microtubules enhance the non-cell-autonomous movement of both endogenous miRNAs and exogenous artificial miRNAs by preventing their loading onto cytoplasmic AGO1 in the source tissues (Fan et al., 2022). amiR-SUL: artificial microRNA targeting *SULFUR* transcript, WT: wild-type, *ktn1:* KATANIN 1 mutant.