RNAs - A New Frontier in Crop Protection

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Abstract

Small RNA (sRNA)-mediated RNA interference (RNAi) is a regulatory mechanism conserved in almost all eukaryotes. sRNAs play a critical role in host pathogen interactions either endogenously or by traveling between the interacting organisms and inducing “cross-Kingdom RNAi” in the counterparty. Cross-kingdom RNAi is the mechanistic basis of host-induced gene silencing (HIGS), which relies on genetically expressing pathogen-gene targeting RNAs in crops, and has been successfully utilized against both microbial pathogens and pests. HIGS is limited by the need to produce genetically engineered crops. Recent studies have demonstrated that double-stranded RNAs and sRNAs can be efficiently taken up by many fungal pathogens, and induce gene silencing in fungal cells. This mechanism, termed “environmental RNAi”, allows direct application of pathogen-gene targeting RNAs onto crops to silence fungal virulence-related genes for plant protection. In this review, we will focus on how we can leverage cross-kingdom RNAi and environmental RNAi for crop disease control.

Introduction

Crops are constantly under siege by pathogens and pests in both the pre- and post-harvest stages, leading to the loss of approximately 30% of crops worldwide (1). Currently, these diseases and pests are largely controlled by chemical pesticides and fungicides, which can leave harmful residues in the environment. Further, overuse of fungicides has led to the development of resistant fungal strains against every major fungicide used in both agricultural and clinical applications(2). Therefore, there is an urgent need to develop innovative, effective and environmentally friendly crop protection strategies to safeguard both global food security and human health.

Existing mechanisms in host-pathogen interactions often serve as a guide for developing novel disease management strategies. Recent advances have identified RNA interference (RNAi), a regulatory mechanism largely conserved throughout Eukaryotes (3), as a critical regulatory mechanism of host immunity (4), pathogen virulence (5), and host-pathogen communication (6, 7). RNAi generally suppresses gene expression via small RNAs (sRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs) (3). These sRNAs are generated by Dicer or Dicer-like (DCL) proteins and loaded into Argonaute (AGO) proteins and silence genes with complementary sequences to the guide sRNA (3). In plants specifically, RNAi plays a critical role in the regulation of gene expression in response to infection of pathogens and pests(4).

Emerging discoveries have revealed that sRNAs, in addition to their endogenous functions, are also transported between hosts and their pests/pathogens, where they can induce “cross-kingdom or cross-species RNAi” in the counterparty (5, 8–10). Cross-kingdom RNAi makes it possible to apply host-induced gene silencing (HIGS) for plant disease control. In HIGS, host plants are genetically engineered to express pathogen- or pest-gene targeting double-stranded RNAs (dsRNAs) or sRNAs. These RNAs are then transported into the pest or pathogen via Cross-Kingdom RNAi, where they target and silence pest or pathogen genes, conferring protection to the plant host (11).

Additionally, recent research has found that some eukaryotic pathogens,

such as some nematodes and many aggressive fungal pathogens, are capable of taking up RNAs from the environment (6, 12, 13). The transferred dsRNAs and sRNAs that have complementary sequences to the genes in the organism can potentially induce silencing of the target genes, a phenomenon named "Environmental RNAi" (12). This discovery prompted the development of Spray-induced gene silencing (SIGS), where artificially synthesized pathogen or pest gene-targeting dsRNAs or sRNAs are sprayed directly onto plant material. These RNAs then target and silence pathogen genes through Environmental RNAi, inhibiting disease development **(Figure 1)**(13–15). Current research efforts are focusing on utilizing nanomaterials to stabilize the RNA on plant material and enhance the delivery of these RNAs to the target pathogens (16).

In this review, we will summarize the function of cross-kingdom RNAi in plant and microbe interactions, discuss the advantages and limitations of both HIGS and SIGS, and focus on the development of new strategies to improve the application efficiency of SIGS for disease control in agriculture.

Cross-Kingdom RNAi

Recent studies have revealed that, in addition to their endogenous functions, sRNAs can travel between hosts and interacting organisms to silence target genes within interacting organisms, through Cross-Kingdom RNAi (5–7, 10, 17, 18). Cross-Kingdom RNAi was initially observed in plant-fungal interactions (5). Specifically, the fungal pathogen *Botrytis cinerea*, the causal agent of gray mold on hundreds of plant species, delivers a panel of sRNAs into various plant hosts, such as *Arabidopsis thaliana* and tomato, and hijacks the key component of host RNAi machinery, AGO1, to silence plant immune response genes (5). These fungal sRNAs serve as a novel class of pathogen effector molecules to suppress host immunity. Since this initial discovery, other plant fungal pathogens, such as *Verticillium dahliae* and *Puccinia striiformis,* have been found to transport sRNAs into their plant hosts to silence defense response genes (6)(18). *V. dahliae* sRNAs were also found to be loaded into host Arabidopsis AGO1 for RNAi (6).

Further studies in the *Arabidopsis* and *Botrytis* plant-pathogen system have shown that cross-kingdom RNAi can be bi-directional (6, 7). *Arabidopsis*

also delivers sRNAs into *B. cinerea* and silence fungal virulence-related genes, such as Dicer-like proteins (DCLs) that generate sRNA effectors, and genes that regulate vesicle trafficking (*Bc-VPS51*, encodes for vacuolar protein sorting 51; *DTCN1,* encodes the large subunit of the dynactin complex, *SACI,* that encodes a phosphoinositide phosphatase), to inhibit fungal virulence (7).

Since these initial studies in plant-fungal interactions, cross-kingdom/cross-species RNA trafficking has been observed in a variety of interaction systems. For example, the parasitic plant, *Cuscuta campestris*, sends miRNAs into its host plants to silence plant defense genes (19). Even the prokaryotic *Rhizobium*, a symbiotic bacterium, can transport transfer RNA (tRNA)-derived sRNA fragments into the soybean cells to silence nodulation-related genes using host AGO1 (20). In response to infection, cotton plants can also send specific miRNAs to *Verticillium dahliae* infection and silence essential fungal virulence genes (21). Similarly, *Arabidopsis* plants deliver sRNAs into the oomycete pathogen *Phytophthora capsici* and suppress expression of *P. capsici* genes, leading to a decrease in mycelial growth and defective sporangia development (17). Furthermore, Dunker et al. discovered that *Hyaloperonospora arabidopsidis*, a plant oomycete pathogen that is phylogenetically distant from fungi, can also send sRNAs into host plants and utilize host AGO1 protein to silence plant genes (22).

The phenomenon of sRNA trafficking from infectious organisms to hosts has also been observed in animal-pathogen/parasite interaction systems (10, 23, 24). For example, the gastrointestinal nematode secrete exosomes containing miRNAs to modulate the immune response of infected mammalian hosts (10). Strikingly, even the functional molecular mechanism of some fungal sRNAs in animal hosts is conserved as in plants. A mosquito fungal pathogen *Beauveria bassiana* delivers an miRNA to mosquito cells and also employs host AGO1 to silence mosquito gene *Toll receptor ligand Spätzle 4 (25)*.

Though a precise pathway for sRNA transport between organisms remains to be elucidated, several studies have demonstrated that extracellular vesicles (EVs) are the main mechanism by which sRNA moves between cells of different organisms (7, 17, 26, 27). Further, Cai et al. further demonstrated that a specific class of EVs, the tetraspanin-positive exosomes, are mainly

responsible for sRNA transport (7). The sRNA cargo is not simply loaded into EVs through concentration-dependent diffusion, but an active selection process is involved. Specifically, He et al. have recently shown that several RNA-binding proteins, which are also loaded into plant extracellular vesicles, contribute to both selective sRNA loading into EVs and sRNA stabilization once inside the EVs (28). These RNA-binding proteins include the key component of RNAi machinery, AGO1, which binds to sRNAs to induce RNAi of target genes with complementary sequences, a subfamily of DEAD-box RNA Helicases RH11, RH37 and RH52, and annexins (28). Understanding the mechanisms of naturally occurring RNA-based communication between plants and their pathogens has aided in the development of novel crop protection strategies.

Host-induced gene silencing— HIGS

Due to its prevalence across plant-pathogen interaction systems, the principle of cross-kingdom RNAi is utilized in crop protection strategies, through Host-induced Gene Silencing (HIGS). In HIGS approaches, plants are genetically engineered to produce pathogen/pest gene-targeting sRNAs or dsRNAs that are processed into sRNAs. These sRNAs are subsequently transferred into the pest or pathogen to silence virulence-related genes (29). HIGS is effective against a wide variety of plant pathogens and pests, including viruses, viroids, fungi, insects and nematodes(11, 30, 31). Further, HIGS has been utilized successfully in important crops, including wheat, barley, and soybean to effectively combat various pathogens, such as *Blumeria graminis (32), Puccinia triticina (33), Fusarium graminearum (34)*, and *Phakopsora pachyrhizi (35)*. These examples illustrate that HIGS is a promising tool to limit chemical-based pesticide applications.

Additionally, HIGS is a versatile tool, as the engineered RNA constructs can be designed to target multiple pathogens simultaneously. Wang et al. provided a successful example by producing *Arabidopsis* plants with sRNAs targeting Dicer-like genes (DCLs) in two invasive fungal pathogens such as *Botrytis cinerea* and *Verticillium dahliae*, thus providing protection from both pathogens (6). Moreover, because sRNAs do not need to be completely complementary to their target mRNA for effective silencing (36), single point

mutations in the target gene are unlikely to yield resistant pathogens.

Although HIGS is effective, it involves the generation of genetically modified (GM) crops, which remains technically challenging and time consuming in many crop varieties. Further, regulatory hurdles can rapidly increase the cost and time required to bring a transgenic crop to market (37). Despite these challenges, in 2017, the Environmental Protection Agency (EPA) approved GM corn to express a dsRNA against an insect pest, Western corn rootworm, called SmartStax Pro, which may be released in the United States in the next few years (38).

Spray-induced gene silencing—SIGS

Due to the lengthy and costly process of generating GM crops, a plant-disease management strategy not reliant on transgenic approaches is highly desirable. Recently, Wang et al. demonstrated that the fungal pathogen, *Botrytis cinerea*, could take up environmental RNAs, though the specific RNA uptake mechanism remains unknown(6). This discovery prompted the development of an eco-friendly, GM-free, RNAi-based plant protection strategy, Spray-induced gene silencing (SIGS)(14). In SIGS applications, pathogen-gene targeting RNAs are sprayed directly onto plant materials in order to confer protection. Externally applied sRNAs and dsRNAs targeting *B. cinerea DCL1* and *DCL2* can effectively inhibit *B. cinerea* disease formation on a variety of post-harvest plant materials, including vegetables, fruits, and flowers, as well as on *Arabidopsis* and tomato plants (6, 13). Results in barley demonstrated that application of *Fusarium graminearum* gene-targeting dsRNA prevents the growth of the pathogen (39). SIGS approaches can also inhibit infection of *Brassica napus* by the pathogens *S. sclerotiorum* or *B. cinerea (40)*. More recently, results have demonstrated that SIGS approaches can reduce biomass accumulation of fungal pathogen *Phakopsora pachyrhizi* in soybean by 75% (35). Remarkably, dsRNA applications could control *F. graminearum* growth and infection not only at the local application site, but also in the distal untreated part of the leaf, suggesting that dsRNAs on plant surfaces can be taken up and transported within plant tissues, and that the silencing molecules is transmitted to the distal part (39).

Early successes of SIGS approaches demonstrate the potential for a new class of RNA-based fungicides to be developed. An RNA-based fungicide could offer many key advantages over traditional fungicides. Specifically, because RNA is already present in most food, it is likely to be safe for consumption. Additionally, like HIGS, RNAs developed for SIGS can be designed to target multiple pathogens simultaneously, and because complete base pairing is unnecessary for effective silencing (36), fungicide-resistance strains are less likely to develop. Another key advantage of RNA-based fungicides is that, unlike traditional fungicides which can leave harmful residues in ecosystems, RNAs rapidly degrade in the soil (41). In fact, this rapid environmental degradation is a major hurdle in the practical application of SIGS to control soil-borne pathogens.

The efficacy of SIGS approaches is dependent on the RNA uptake efficiency of the pathogen (13). Many aggressive fungal pathogens can take up RNAs from the environment very efficiently, even as quick as within a couple of hours, which makes it possible to apply SIGS for plant protection against these pathogens (13). In order to improve both RNA stability and RNA uptake efficiency, current efforts are focused on nanoparticle technology to improve the application system and the limited durability of the RNAi effect (42).

Key Considerations for SIGS Strategies

dsRNA fragment properties

The dsRNA sequence used to induce gene silencing should be designed to optimize the gene downregulation in the pathogen. First, sequence design must take into account the secondary structure of the selected target sequence because complex RNA structures can prevent the base-pairing between sRNA and the target and inhibit cleavage of mRNA by the RISC complex (43–45). Second, the siRNA must be designed to avoid secondary structure formation in the guide-RNA, which can considerably reduce the strength of silencing (46). In HIGS applications specifically, designed sRNA that favorably bind to AGO1 have a better chance of being selectively loaded into EVs and subsequently transported to the pathogen or pest (28).

On the other hand, as few as 11 contiguous nucleotides or 15 out 19

base pairs of complementarities can lead to off-target silencing (47). Therefore, it is critical that SIGS RNA constructs are designed to avoid targeting genes in host plants or beneficial microbiota, taking into account both sense and antisense strands since either could potentially serve as the guide RNA strand. To do this, SIGS RNA constructs should be designed to target non-conserved sequences within the target gene, and genome-wide base-pairing analysis should be performed to avoid any base-pairing regions longer than 15 nt with the genomes of the hosts and other beneficial microbes. A final consideration in SIGS RNA construct design is to optimize the RNA length, as the optimal length can vary across pathosystems (48–50).

Pathogen RNA uptake efficiency

RNA uptake efficiency of a pathogen is critical in determining how effective SIGS strategies will be against that pathogen. For example, two pathogens which SIGS strategies are highly effective against, *B. cinerea* and *F. graminearum,* also possess high environmental RNA uptake efficiency (6, 39). Recent findings demonstrate that different types of eukaryotic microorganisms and different cell types within an organism have distinct RNA uptake efficiencies. Specifically, the fungal pathogens *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahliae* have high RNA uptake efficiency, whereas *Colletotrichum gloeosporioides* exhibits no RNA uptake; and the beneficial fungus *Trichoderma virens* has weak rates of RNA uptake (13). Externally applied RNAs that target virulence genes could suppress the disease caused by fungal pathogens that have a high RNA uptake efficiency but could not inhibit diseases caused by pathogens with low environmental RNA uptake efficiency (13). Thus, it is important to examine the RNA uptake efficiency of a particular fungal or oomycete pathogen before you apply SIGS to control this pathogen on plants.

Plant RNA uptake efficiency

In addition to direct uptake by pathogens or pests, RNA can also get inside of plants **(Figure 1)**(6, 39). The efficiency of exogenous dsRNA absorption varies across different plant tissues. Dalakouras et al. found that

high pressure spraying is an effective method to transfer exogenous siRNAs into plant cells to induce RNAi. In fact, leaf and bud spraying is more effective than petiole absorption or trunk injection to induce RNAi (51). Further, damaged plant surfaces have higher dsRNA uptake efficiency than healthy plant surfaces (52). The uptake efficiency of dsRNAs molecules in the spray may be related to the parameters of uptake efficiency, such as stomatal opening (39).

Nanoparticles as carriers of RNAi for crop protection

Inorganic nanoparticles as RNA carriers

The biggest hurdle SIGS technology must overcome before commercial use is the relative instability of RNA in the environment. Currently, research is focusing on the potential for inorganic nanoparticles to enhance RNA stability and pathogen RNA uptake. (53). Nanoparticles as carriers for siRNAs hold great potential for SIGS application. Specific examples of this include layered double hydroxides (LDH) clay nanosheets, guanidine containing polymers and liposome complexes(54–56).

By loading dsRNAs onto LDH clay nanosheets, Mitter et al. developed a technology termed “BioClay”, for use in SIGS applications. BioClay RNAs were not easily washed off plant surfaces, demonstrated sustained RNA release, and remained detectable on treated leaves up to 30 days after application. Further, these BioClays containing virus gene targeting RNAs can provide at least 20 days of plant protection against virus infection (42). A different study tested three nanoparticles, chitosan, carbon quantum dots (CQD), and silica complexed with dsRNA targeting two mosquito genes (*SNF7*, encodes a class E vacuolar sorting protein; and *SRC*, Steroid Receptor Coactivator) for controlling *Aedes aegypti* larvae (57). They found that CQD displayed the most efficient carrier for dsRNA delivery and gene silencing in *Aedes aegypti (57)*.

Some nanoparticles developed for transporting nucleic acids into plant cells are potential candidates for use in SIGS applications. Effective delivery of nanoparticles to plant cells depends on the size and the charge of nanoparticles (58). In comparison to neutral nanomaterials, the charge of nanoparticles with zeta potential higher than 20 or 30 mV are more likely to be absorbed by plant cell membrane or chloroplast membrane respectively. With the decrease of the

size of nanoparticles, a larger zeta potential is needed to make them pass through the cell wall and lipid membrane (58).

Carbon nanotubes have successfully been utilized to transport biomolecules into plant cells, and can provide RNAi payloads for gene silencing by spraying nanotube bound exogenous sRNAs or dsRNAs onto plant surfaces (59, 60). Recently, Demirer et al. developed a nanotube-based platform for siRNA delivery with high silencing efficiency in intact plant cells. The nanotube provided siRNA protection from nucleases and an effective intracellular delivery of the siRNA which resulted in a steady plant RNAi (60). Unfortunately, the documented toxicity of carbon nanotubes to humans and mammals makes them an unideal candidate for SIGS approaches (61).

Alternatively, Schwartz et al. recently established a novel tool for gene silencing in plants by packaging siRNAs in Carbon dots (62). Simple spray application of carbon dots resulted in strong silencing of *GFP* (Green Fluorescent Protein) transgenes in *Nicotiana benthamiana* and *Solanum lycopersicum* but also of two endogenous genes that encode two subunits of the magnesium chelatase protein of the plants (62). Because these carbon dots are able to successfully infiltrate plant cell walls, it is likely that they can also be taken up by walled plant pathogens, such as fungi. Another study established a nanoscale platform to deliver biochemical nanomaterials to plant photosynthetic organelles (chloroplasts) by targeting peptide recognition motifs. In this study, peptide biometrics provided Quantum dot (QD) functionalization with β - cyclodextrin molecular baskets in order to carry cargoes to a specific subcellular compartment with high efficiency (63).

Organic Nanoparticles as RNA carriers

Another potential strategy for RNA delivery to plant pests and pathogens is to mimic naturally occurring RNA transport pathways. Cai et al. found that plant cells secreted EVs containing host sRNAs that can be efficiently taken up by *B. cinerea* fungal cells, suggesting plant EVs are the major mechanism for RNA transport into fungal pathogens (7). Recently, a set of RNA binding proteins, including AGO1, RNA helicases and annexins were found in *Arabidopsis* EVs, and contribute to selectively loading and/or stabilization of

sRNAs into EVs (28). In order to mimic this naturally occurring pathway, it is possible that lipid-based nanovesicles could be developed for RNA delivery to plant pathogens. In fact, lipid nanoparticles isolated from grapefruits can deliver therapeutic agents, including siRNAs, to mammalian cells (64) and liposomes have already been utilized in clinical contexts for delivery of siRNAs in the bloodstream (65). These liposomes can be adapted for use in plant-protection, by loading them with pathogen-gene targeting RNAs to form artificial vesicles. Further, co-delivering key RBPs, such as AGO1, in these artificial vesicles could potentially increase the payload, stability, and silencing efficiency of SIGS RNAs in pathogenic microbes, including those lacking their own RNAi machinery. These lipid-based approaches may also be effective in transporting RNA to plant pathogens.

Finally, another approach to RNA delivery is to genetically engineer bacteria to produce the dsRNA fragment of interest. This approach had already been demonstrated in insects (66), nematodes (67) and mammalian cell cultures (68). In a recent study, the RNaseIII-null mutant strain of *Escherichia coli* generated dsRNA molecules successfully induced RNAi in *Aspergillus flavus (69)*. Although the specific mechanism of RNA transport from bacteria to fungi remains to be determined, studies suggest that bacteria may transfer sRNAs to eukaryotic cells through outer membrane vesicles, which have been successfully used as siRNA-delivery vehicles for cancer therapy in mammalian animal models (70).

Conclusion

In today's world, crop producers are tasked with producing more food than ever for a constantly growing population, while a changing climate puts stressed crops at higher risk for pathogen attack. As resistance to traditional chemical disease control measures increases at an alarming rate (2), it is becoming clear that innovative plant disease control strategies are critical to maintaining global food security. In addition to agricultural uses, RNA-based disease therapeutics are being developed for use in humans (71), and the first sRNA drug, ONPATTRO (72), was approved for clinical use in 2018. Further,

two of the widely used vaccines against COVID-19, manufactured by Pfizer and Moderna, are mRNA vaccines (73). In parallel to these clinical developments, RNA based technologies potentially represent the next generation of crop protection strategies.

The RNAi based approaches, HIGS and SIGS, both offer flexible and environmentally friendly solutions for crop protection. Though HIGS is limited by the expense and time associated with transgenic crop generation, currently, SIGS is able to circumvent this problem through direct application of RNAs onto plant tissue. Further, SIGS applications can potentially contain a combination of different dsRNA molecules, a mixture of dsRNA and siRNA molecules, or a combination with insecticides or fungicides to enhance plant protection, target multiple pathogens simultaneously, and prevent the emergence of resistant or tolerant mutant pathogens (14, 74). Currently, the focus of SIGS research is developing nanoparticle-based delivery systems to both enhance RNA stability on plant tissue, which will reduce the application frequency for growers, and, enhancing RNA uptake, which will potentially lower the amount of RNA needed per treatment. Further, any RNA delivery strategy must be both cost-effective and user-friendly for use in agricultural applications. Overall, the RNAi technologies described here represent an innovative approach to crop disease management that will help ensure global food security moving into the future.

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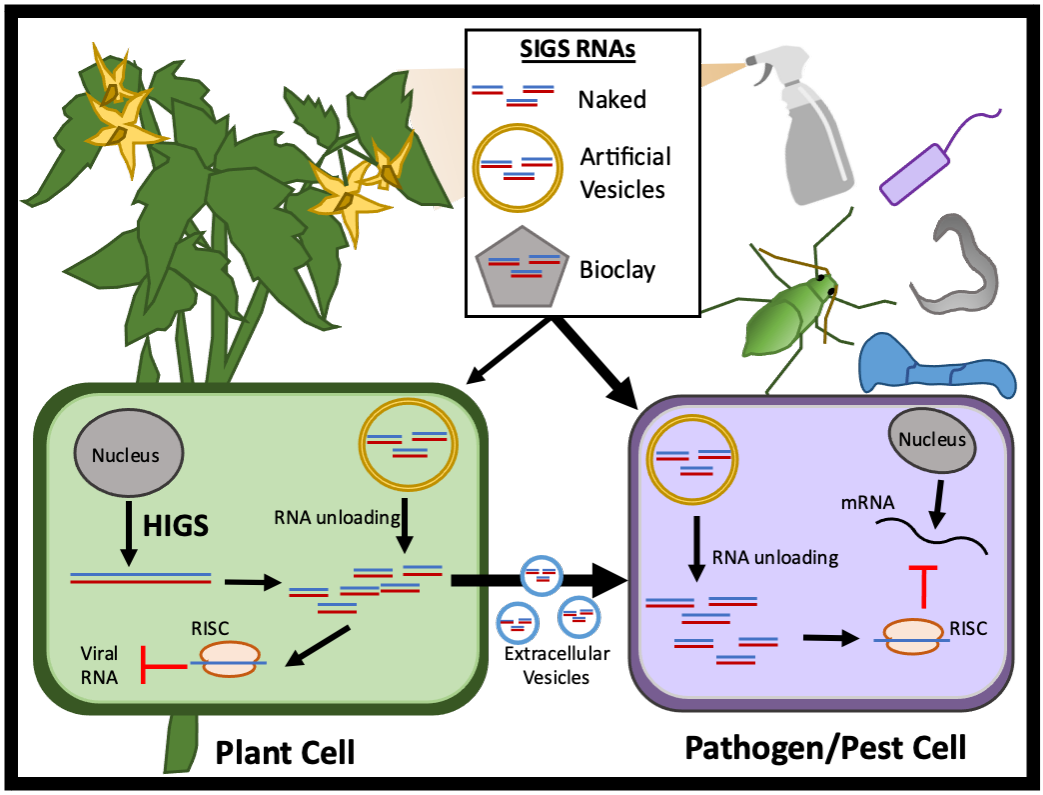

Figure 1: RNAi-Based Plant Protection Strategies. Two main strategies for RNAi based plant disease control exist, Host-Induced Gene Silencing (HIGS) and Spray-Induced Gene Silencing (HIGS). In HIGS approaches, the genetically engineered plant encodes pathogen targeting, double-stranded RNA in the nucleus, which is transported to the cytoplasm, where it can be processed into small RNAs by DCL proteins. These small RNAs can then be transported into pathogen or pests, via extracellular vesicles, where they target and silence pathogen mRNAs. Alternatively, they can operate within the plant cytoplasm to target and silence viral RNA. In SIGS approaches, pathogen targeting RNA, naked, packaged in organic nanoparticles, or packed in inorganic nanoparticles, is sprayed directly onto plant tissue. Next, it can be taken up by the pathogen/pest, where it targets and silences pathogen/pest genes. Alternatively, these sprayed RNAs can first be taken up by the plant, and then subsequently transported into the pest or pathogen.