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Improving RNA-based crop protection through nanotechnology and insights from cross-kingdom RNA trafficking

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Abstract

Spray-induced gene silencing (SIGS) is a powerful and eco-friendly method for crop protection. Based off the discovery of RNA uptake ability in many fungal pathogens, the application of exogenous RNAs targeting pathogen/pest genes results in gene silencing and infection inhibition. However, SIGS remains hindered by the rapid degradation of RNA in the environment. As extracellular vesicles are used by plants, animals, and microbes in nature to transport RNAs for cross-kingdom/species RNA interference between hosts and microbes/pests, nanovesicles and other nanoparticles have been used to prevent RNA degradation. Efforts examining the effect of nanoparticles on RNA stability and internalization have identified key attributes that can inform better nanocarrier designs for SIGS. Understanding sRNA biogenesis, cross-kingdom/species RNAi, and how plants and pathogens/pests naturally interact are paramount for the design of SIGS strategies. Here, we focus on nanotechnology advancements for the engineering of innovative RNA-based disease control strategies against eukaryotic pathogens and pests.

Introduction

Plant pathogens and pests are a significant and devastating problem for the agricultural industry, resulting in average crop losses between 10 and 40% across major food security crops, even with conventional disease mitigation methods [1]. Due to global warming, the range of ecological niches and spread of plant-pathogenic fungi and pests are increasing [2]. Therefore, the development of novel, robust, and eco-friendly antifungals and insecticides is urgently needed.

Effective pathogen control strategies have been developed by exploiting naturally occurring phenomena in host–pathogen interactions. RNA interference (RNAi) has been identified as

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a critical regulatory mechanism in eukaryotes in which small RNAs (sRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs), silence the expression of target genes with certain complementary sequences. sRNAs are processed by the Ribonuclease III-like enzyme Dicer or Dicer-like (DCL) proteins and loaded into Argonaute (AGO) proteins to enable silencing of target genes by mRNA cleavage and degradation, translational inhibition, or transcriptional gene silencing [3-5].

In addition to endogenous regulation, sRNAs are transported between interacting organisms in a mechanism deemed “cross-kingdom RNAi”. Cross-kingdom RNAi is part of plant defense responses in which plants produce and transfer sRNAs to silence pathogen genes and inhibit infection. Host-induced gene silencing (HIGS) is to engineer plants to express double stranded RNAs (dsRNAs) or sRNAs that target essential or virulence-related genes in pathogens/pests [6]. Although effective, generation of HIGS-modified crops is time-consuming, challenging, and unfeasible for some plant species. HIGS plants are also classified as genetically modified organisms, which have an arduous regulatory approval process and are unfavorable in some consumer markets [6,7].

Recently, it was discovered that many organisms, including fungi and oomycetes, can efficiently take up RNA from the environment [8]. Exogenous dsRNAs can be processed by native host or pathogen/pest RNAi machinery into siRNAs that silence genes in the pathogen or pest [9-11], a process known as ‘environmental RNAi’. This prompted the development of spray-induced gene silencing (SIGS), in which sRNA or dsRNA targeting pathogen/pest genes are topically applied to plant surfaces for specific and effective crop protection. Since then, dsRNA has been applied for plant protection through various methods including spraying, infiltrating, root soaking, spreading on leaves, and injection with success [11,12]. In addition to the targeting of pathogen, pest, and viral sequences, SIGS approaches have been used to modify endogenous plant genes and phenotypes [13-15]. This ability to alter gene expression can provide a powerful tool for investigating plant genetics.

Although promising, sRNA and dsRNA molecules have a short period of environmental stability and variable uptake rates in pathogens and pests [8]. To improve the efficacy of SIGS, recent advances have been made in nanoparticle-based protection of RNAs that drastically increase the feasibility of RNA-based crop protection strategies. Here, we discuss important considerations for generating successful SIGS strategies against plant pests and pathogens that emphasize both nanoparticle and dsRNA attributes. These include analysis of effective gene targets and their pathways, and new insights into nanoparticle characteristics and dsRNA properties that highly influence cellular uptake and silencing efficiency.

The conserved nature of cross-kingdom RNAi

Cross-kingdom RNA trafficking is an important facet of host–microbe interactions as shown by numerous discoveries of functional RNA transfer between interacting species. In the plant kingdom, cross-kingdom RNAi was observed between the model plant *Arabidopsis thaliana* and the fungal pathogen *Botrytis cinerea,* in which fungal sRNAs enter host cells and hijack *Arabidopsis* AGO1 to silence plant immunity genes, weakening host immunity to promote fungal infection [16]. Like *B. cinerea,* other plant pathogens such as *Fusarium*

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oxysporum [17], *Verticillium dahliae* [18,19], the oomycete *Hyaloperonospora arabidopsidis* [20], and the rust fungus *Puccinia striiformis* [21,22] all send sRNAs into plant host cells to silence host immunity target genes and facilitate infection. Conversely, plant hosts such as *A. thaliana* [23], cotton [24], tomato [25,26], and wheat [27] transport sRNAs to silence pathogen virulence-related genes and inhibit infection, emphasizing the bidirectional nature of cross-kingdom RNAi.

In addition, the mechanisms of cross-kingdom RNAi seem heavily conserved. Many of the sRNA effectors from plant pathogens utilize host AGO proteins, such as AGO1 and AGO4, to silence plant immunity genes as evidenced by studies of *B. cinerea* [16], *V.dahliae* [18,19], *F. oxysporum* [17,28] and *H. arabidopsidis* [20]. Moreover, the parasitic plant *Cuscuta campestris* delivers sRNAs into host plants during parasitism [29]. Cross-kingdom RNAi occurs in symbiotic plant-microbe relationships as well. The ectomycorrhizal fungus *Pisolithus microcarpus* delivers a microRNA into the host *Eucalyptus grandis* and induces cross-kingdom gene silencing during symbiosis [30]. Strikingly, although bacteria lack traditional RNAi machinery, tRNA-derived RNA fragments (tRFs) from nodule-forming Rhizobia bacterium are transferred into host soybean root cells, bind to soybean AGO1, and induce silencing of plant host nodulation-related genes [31].

Cross-kingdom RNAi has also been observed in several animal pathogen/parasite interactions such as between the nematode parasite *Heligmosomoides polygyrus* and mouse cells [32] and between the yeast pathogen *Candida albicans* and human monocyte cells [33]. As well, the insect pathogenic fungus *Beauveria bassiana* transfers sRNAs that bind to host mosquito AGO1 to silence host genes [34]. Cross-kingdom RNAi is conserved among a wide range of interacting organisms, and many microbial sRNAs function similarly by loading into host AGO proteins to silence host genes, either to promote infection or maintain symbiosis. Despite the prevalence of RNA translocation between species, how these RNAs move through extracellular spaces in different organisms is not yet fully understood.

Plant extracellular vesicles are a critical mechanism for sRNA transport

There is significant evidence for the role of extracellular vesicles (EVs) in RNA translocation between interacting organisms. In mammalian systems, diverse cell types including monocytes [33], adipocytes [35], cancer cells [36,37] and immune cells [38,39] all use EVs to protect and transport sRNAs, mRNAs, and other RNAs to specific target cells. Plant fungal pathogen *B. cinerea* also transports sRNAs in fungal EVs, which are taken up by *A. thaliana* cells through clathrin-mediated endocytosis [40]. Furthermore, bacteria, algae, and nematodes utilize EVs for RNA transport to interacting microbes or host tissues [32,41,42]. In plants, the presence of many ribonucleases in the apoplast [43] suggests that extracellular RNAs must have a means of protection. Indeed, *Arabidopsis* EVs, especially TET8-positive EVs have been shown to protect their RNA cargo from degradation from both proteases and RNases, illustrating how plant EVs are an efficacious route of RNA protection and transport within plants [23,47]. It has been speculated that RNA-binding proteins may protect RNAs from degradation. However, most RNA binding proteins only bind to specific motifs of RNA, which is evidenced by methods that use RNase treatment to cleave RNA in RNA-protein complexes to identify RNA-binding sites [44].

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The apoplast also contains many proteases that can disrupt the stability of RNA-protein complexes [45,46]. Therefore, it is questionable whether protein binding is a prevalent or sufficient strategy for extracellular RNA protection in plants. Similar observations in plant, microbial and mammalian cell systems demonstrate how conserved the use of EVs for RNA protection and transport is across kingdoms [37,40,48-50].

The isolation of high quality EVs is critical for studying EVs and their RNA cargo. Three methods have been used for plant EV isolation: differential centrifugation followed by ultracentrifugation, density gradient ultracentrifugation, or immunoaffinity capture. Of the three methods, differential centrifugation followed by ultracentrifugation results in the highest yield of EVs while the other two methods enable isolation of specific EV densities or subtypes. Importantly, the use of swinging-bucket rotors during ultracentrifugation is recommended for collecting high-quality intact EVs, as this type of rotor minimizes vesicle disruption [35,36]. Two ultracentrifugation speeds have been used to pellet plant EVs: 40,000 $\times g$ (P40 fraction) and 100,000$\times g$ (P100 fraction) (Figure 1). The P40 fraction enriches for PEN1-positive EVs, which contain tiny RNAs and proteins [51,52], and non-vesicular RNAs with unknown mechanisms of degradation protection [43,45,46]. Centrifugation of the supernatant of P40 at 100,000$\times g$ (P100–P40 fraction) collects TET-positive EVs, which are enriched in functional sRNAs and proteins [23,47,53]. In density gradient ultracentrifugation, different EV subtypes are separated by density [47,51]. Most reliably, immunoaffinity purification can isolate specific EV subtypes by using antibodies against EV subtype-specific protein markers [46,47]. Unfortunately, few plant EV markers have been characterized to date, so there is a strong need to identify additional markers.

Differences in plant conditions and extraction methods of apoplastic washing fluid can greatly affect the EVs that are captured. Collection of apoplastic washing fluid from detached leaves rather than whole plants alone can reduce contamination from cell debris and cytoplasmic content [47]. Therefore, it is important to avoid generalizations about the function of all plant EV subtypes based on individual methods of capture. Studies of native extracellular RNA protection are paramount in the design of effective RNA application for SIGS. Indeed, mimicking plant EVs using artificial vesicles to protect dsRNA was shown to prolong SIGS-based plant protection against *B. cinerea* [54].

Leveraging RNAi to control plant pathogens and pests

Fungi and oomycetes are highly amenable to RNAi-based control strategies. More than 50 proof-of-concept studies have demonstrated the viability of HIGS-mediated control of ascomycetes, basidiomycetes, and oomycetes, with an average disease resistance of 60% [6]. The success of HIGS is positively correlated with the quality and quantity of sRNA transport from plant cells [55] and uptake by pathogens/pests [6]. Efforts to elucidate these mechanisms are ongoing. Sustainably produced dsRNA/siRNA in HIGS leads to long-lasting RNAi, which is important for organisms that do not have RNAi amplification or transitivity mechanisms [56]. HIGS also eliminates the need for repeated dsRNA application as is the case with SIGS [57]. However, for some phloem-sucking insects and necrotrophic pathogens, dsRNA processing by plant RNAi machinery can limit HIGS efficacy [58,59]. Several studies have highlighted how dsRNA is more effective for gene silencing and

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inducing phenotypic changes in pests/pathogens than siRNA [60,61]. As such, SIGS has been demonstrated to have similar or higher efficacy than HIGS against some fungi and insects [61-63]. It may be partially due to the lower stability of sRNAs than long dsRNAs.

The generation and secretion of sRNA effectors is important for virulence in many fungal and oomycete pathogens, so RNAi pathway components, such as *DCL* genes, represent effective SIGS targets. Although a few examples of Dicer-independent sRNA biogenesis in fungi and mammals have also been reported [64-66], successful disease control through SIGS of pathogen DCL genes has been demonstrated with *B. cinerea* [19,67], *Fusarium graminearum* [68,69], and *Plasmopara viticola* [70]. As well, mutation of fungal *DCL* genes attenuated the virulence of *F. graminearum* [69], *Penicillium italicum* [71], *P. viticola* [70] and *Valsa mali* [72], and both the virulence and growth of *B. cinerea* [16,19,67] and *Colletotrichum gloeosporioides* [73]. Fungal-derived phased siRNAs were recently discovered at the *Blumeria hordei*-barley interface, suggesting a role in endogenous sRNA-directed fungal gene regulation through the fungal RNAi pathway during infection [74]. Phased siRNAs are mostly generated by DCLs and RNA-dependent RNA polymerases (RdRPs), suggesting that RdRPs could be good potential targets for SIGS. However, in SIGS against *Fusarium asiaticum* [75] and *V. dahliae* [56], RdRPs were found to be less critical for secondary amplification than in plants, indicating the importance of targeting genes from other pathways in tandem. Genes involved in fungal vesicle trafficking such as vacuolar protein sorting 51 (*VPS51*), dynactin complex large subunit (*DCTN1*), and suppressor of actin (*SAC1*) [54,76], as well as other essential genes for fungal growth and development have emerged as effective dsRNA targets [9,77].

Improving RNAi-based plant protection through smarter dsRNA designs

Selecting the appropriate target genes for HIGS or SIGS is the most essential factor for successful pest/pathogen control; however, additional features such as dsRNA stability, transport, length, and processing are key for improving efficacy. Clathrin-mediated endocytosis (CME) is a primary mechanism for dsRNA uptake in fungal pathogens and insect pests [78-80]. Consequently, it has become apparent that chimeric dsRNA designs, rather than dsRNA cocktails, are more effective for SIGS and should be considered for use in future studies [81-83]. Chimeric dsRNAs avoid the potential oversaturation of endocytic components and competitive inhibition of target genes observed during combinatorial delivery of multiple dsRNAs [82] and can enable multiplexed targeting of different genes, pathways and organisms in a single construct [81,83].

Co-opting clathrin-independent endocytosis pathways in addition to CME can further drive SIGS improvement. Design of short (23 nt) “paperclip” RNAs with partially closed ends bypassed CME inhibition in the yellow fever mosquito *Aedes aegypti* to enter cells via a clathrin-independent pathway and facilitate transcript knockdown [79]. Suggested resistance mechanisms that pests/pathogens may evolve to evade SIGS includes mutations of RNA uptake pathways. Therefore, accessing multiple uptake routes is important. Chemically modifying dsRNA can affect how it is enzymatically processed, leading to increased resistance to nuclease degradation and improved RNAi [84]. dsRNA lengths of ~500 nucleotides are also the most optimal for SIGS in balancing efficient cellular uptake/

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processing and minimizing potential degradation and off-target effects [85,86]. Off-target RNAi has been observed with dsRNA that contains matching sequences of 29–32 bp with non-target genes [87], so rational design of dsRNA sequences to minimize these effects and maximize silencing efficiency is of utmost importance.

Nanoparticles as RNA carriers for protection

The vulnerability of dsRNA to environmental factors (e.g., rainfall, UV light), enzymatic degradation, and undesirable processing by the plant RNAi machinery substantially limits SIGS as a technology [88-90]. However, nanoparticles, which are materials with dimensions between 1 and 100 nm in diameter, can assist in overcoming these challenges and are becoming increasingly common in plant protection schemes. Nanoparticles can also be designed with unique physicochemical and biological properties that further augment the efficacy of SIGS (Figure 2).

For some pathogens and pests, undesirable processing of dsRNA into siRNA by plant machinery can result in lower HIGS and SIGS efficacy. Several examples have demonstrated how direct application of exogenous RNA is sufficient for targeted suppression of transgenes in *Arabidopsis* [91,92]. Sprayed dsRNA can be internalized by plant cells and processed into siRNAs by plant RNAi machinery or left intact and translocated to other tissues [93]. It is currently unknown what factors dictate the amount of internalized dsRNA that is translocated rather than processed into siRNAs. In RNAi efforts against agricultural pests, noted variations in RNAi efficacy have been attributed to differences in the levels of unprocessed dsRNA available for pest/pathogen uptake [59]. Plant chloroplasts lack RNAi machinery and accordingly, the use of chloroplast-mediated RNAi was more effective against chewing [94] and nonchewing insects [95] versus traditional HIGS approaches using nuclear-expressed dsRNA. Artificial miRNAs containing insect pre-miRNA backbones also eluded processing by plant Dicer, culminating in enhanced gene silencing and mortality of *Helicoverpa armigera* relative to pre-miRNAs with plant backbones [96].

Furthermore, it has been shown that necrotrophic fungi, at least *F. graminearum,* can take up long, unprocessed dsRNA precursors from plant tissues in addition to the dsRNAs sprayed on the surface [93,97]. This may explain why SIGS is more effective than HIGS in the barley-*F. graminearum* pathosystem [61,86]. Like in some insects, dsRNA must be processed by pathogens to result in gene silencing [97,98]. Supportively, mutation of fungal *DCL* genes in *F. graminearum* desensitized the fungus to dsRNA-mediated gene silencing, but susceptibility to siRNAs was unaffected [97]. For pathogenic fungi, nitrogen and phosphate fertilization has been shown to promote their growth relative to mutualists and saprotrophs [99]. Several bacterial species can also utilize extracellular DNA as a carbon and phosphate source [100,101]. Therefore, fungal pathogens could be motivated to take up exogenous RNA as nutrients through mechanisms such as endocytosis [78], although this remains to be experimentally demonstrated.

Use of nanoparticles to stabilize dsRNA can prevent detrimental dsRNA internalization, enzymatic degradation, and processing by plant cells [45,46]. In several cases, RNA release from nanoparticles is facilitated by acidic conditions that are induced by pathogens upon

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invasion, enabling direct and targeted delivery of dsRNA to the pathogen [102,103]. Moreover, nanoparticle compositions can be engineered for RNA delivery to specific tissues and organs [104-106]. Efficient RNA delivery can also be achieved with non-cell-internalizing nanoparticles as siRNA can be released from the nanoparticle surface by surrounding biofluids [107]. Therefore, nanoparticles can improve RNAi efficacy by increasing the local concentration and residence time of dsRNA available for cellular uptake by preventing its cytoplasmic processing or degradation.

Use of nanoparticles in insects overcomes endosomal accumulation and extreme pHs

Efforts to control insect pests using naked dsRNA have had variable results due to the challenges of RNA instability, limited cellular uptake, overcoming endosomal accumulation after ingestion, and extreme pH modalities in the insect that lead to dsRNA degradation. As such, encapsulation of dsRNA in nanoparticles can improve SIGS efficacy by addressing these challenges. This is critical since dsRNA is often taken up by cells through endocytosis, and if it cannot exit endosomes, the dsRNA will eventually be degraded in lysosomes, resulting in inefficient RNAi [108,109]. Consequently, RNA loaded into layered double hydroxide (LDH) clay nanosheets [81,110], chitosan [111-113], carbon-based materials [114,115], liposomes [116-118] or star polycations [119,120] resulted in more severe physical deformations, retarded larval growth, and increased mortality up to 90% relative to naked dsRNA. Nanoparticle use further critically prevented salivary degradation of dsRNA [117,118,121] and degradation in pHs up to 11 [122]. Although for some insects, additional surface modifications are required to facilitate effective SIGS. In *A. aegypti,* conjugation of chitosan nanoparticles with sodium tripolyphosphate (TPP) was required to induce a 20–65% mortality rate [123]. Addition of a phenolic coating on dsRNA-biodegradable polymers similarly enhanced endosomal escape and increased cellular uptake in *Spodoptera frugiperda* (Sf9) cells to facilitate RNAi [118]. These modifications imparted greater size control, a more positive charge, and stronger dsRNA binding that provided greater stability and internalization [118,123]. Nanoparticle-dsRNA treatment can also upregulate endocytosis-related genes in plant [124] and insect [125] cells relative to naked dsRNA, which further contributes to the enhanced RNA delivery and gene silencing effects observed.

Nanoparticles in SIGS against microbial pathogens

Although not as extensively studied as insects, progress is being made in utilizing nanoparticles in SIGS-based control of fungal and microbial pathogens [54,76,126-128]. Topical application of LDH containing dsRNA targeting sRNA effector biogenesis genes DCL 1 & 2 or vesicle trafficking genes *VPS51, DCTN1,* and *SAC1* (VDS) genes extended pathogen protection on pre-harvest materials for up to 4 weeks and on postharvest materials for up to 10 days [54,76,129]. Nanoparticle use can also overcome the constraint of limited RNA uptake rates shown by a subset of fungal/oomycete pathogens [8], which extends SIGS-based control strategies to previously inaccessible organisms. Using carbon dots (CD), Wang et al. enabled SIGS-based control of wild-type and fungicide-resistant *Phytophthora* pathogens by improving dsRNA stability, release, and internalization through CME [127].

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In *Rhizoctonia solani*, star polymer-complexed chitosan nanoparticles outperformed other formulations, including unmodified chitosan nanoparticles, in terms of dsRNA stability and cellular uptake with plant protection observed up to 20 days later [128]. Importantly, these efforts highlight the importance of nanoparticles and their surface chemistry for extending SIGS as a control strategy to a broader range of pests/pathogens.

Metal nanoparticles have previously been used alone or in combination with conventional fungicides to control fungicide-sensitive and -resistant pathogens such as *B. cinerea* and *Alternaria alternata* [130-132]. Therefore, combining these nanoparticles with RNA delivery could enable multipronged control of fungal pathogens by integrating RNAi-based control with traditional fungicides. In voriconazole fungicide-resistant *Aspergillus flavus,* treatment with Lipofectamine™3000 +siRNA complexes lowered the minimum inhibitory concentration of voriconazole 2- to 4-fold [133]. Similarly, complimenting fungicide application with dsRNA-CD treatment reduced the amount of fungicide needed to achieve protection from *Phytophthora* by 90% compared to the fungicide alone [127].

Systemic and sustained protection can be achieved by tuning nanoparticle properties

In 2017, a seminal study demonstrated how dsRNA-LDH application enabled dsRNA persistence on plant leaves for up to 30 days and protection against plant viruses for up to 20 days on sprayed and newly emerged leaves [102]. Concomitantly, a single foliar spray of lipid-modified polyethylenimine (lmPEI) loaded with siRNA targeting grapevine leafroll associated virus-3 (GLRaV-3) was sufficient to reduce GLRaV-3 titers in grapevine. Furthermore, multiple doses of the treatment could facilitate recovery of infected grapevines and berries [134]. This ability of SIGS to provide protection to newly emerged and distant plant tissues is critical for long-term protection and is supported by studies demonstrating long-distance trafficking of RNA through the vasculature [135]. Therefore, understanding how nanoparticle-RNA complexes are transported within plants and taken up by plant pathogens and pests will greatly advance the development of systemic RNAi-based protection methods.

Investigations on LDH, CDs, CeO_2, and silica nanoparticles have all illustrated how small (<50 nm), hydrophilic, and more positively charged nanoparticles exhibited the highest foliar delivery efficiencies into organelles, diverse cell types, and the extracellular space [105,136,137]. As such, size-dependent silencing effects can be observed after spray application with carbon dots [138]. Furthermore, foliar application of smaller (~6 nm) star polymer nanocarriers favored symplastic unloading in young leaves, while larger particles (~35 nm) improved apoplastic unloading in roots [139]. Modulating nanoparticle compactness and bending stiffness can enable nanomaterials to easily move through the plant cell wall to further improve plant uptake [107,140]. Finally, nanoparticle zeta potential (ζ) strongly influences gene silencing efficiency and impacts internalization and mobility as well. Conditions that induced a net negative zeta potential of chitosan-dsRNA or star polycation complexes, including high pH [>7] media conditions or high natural organic

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matter, abolished dsRNA-mediated gene knockdown in *Caenorhabditis elegans* [141] and limited nanocarrier mobility to young leaves and roots [139].

Many of these nanoparticles are likely translocated via the vascular system through energy-dependent and independent processes, as their small size allows them to pass through the plant cell wall [105,137]. This mechanism also extends to trees, where trunk injection of fluorine- and ruthenium-labelled polymer nanoparticles resulted in their translocation throughout the trunk tissue via the xylem sap [142]. Therefore, targeted protection of specific plant tissues will be achieved by designing nanomaterials that promote translocation through the plant vasculature or co-opt specific uptake routes. For example, spray application of siRNA complexed with cell-penetrating peptides enabled efficient and targeted gene silencing through stomata-dependent-uptake and delivery to plant nuclei and chloroplasts [106].

Bacterial-based systems facilitate sustained SIGS in pests and pathogens

In contrast to the nanomaterial systems described above, the use of bacterial-based systems for RNAi can allow for simultaneous synthesis and delivery of dsRNA. This can lower the cost of SIGS by providing sustained dsRNA delivery, eliminating the need for repeated dsRNA application. Furthermore, since insects lack RdRPs, engineering bacterial symbionts for dsRNA delivery can provide sustained RNAi throughout the host's life and overcome challenges associated with feeding or injecting dsRNA. Bacterial-based RNAi already shows great promise against viruses and fungal pathogens with topical treatment of *Escherichia coli*-encapsulated dsRNA, or the dsRNA-producing bacteria itself, protecting plant material against infection as effectively as chemically synthesized naked dsRNA [143,144]. In addition, *in vitro* application of *E. coli*-derived anucleated minicells loaded with dsRNA targeting chitin synthase class III (Chs3a, Chs3b) and the DCL1 and DCL2 genes of *Botryotinia fuckeliana* halted disease progression on strawberries for up to 12 days in greenhouse conditions [145]. Efforts to utilize bacterial symbiont-mediated RNAi (smRNAi) for insect control, however, have yielded mixed results.

In thrips and kissing bugs, smRNAi was effective in reducing gene expression and providing greater protection of cucumber seedlings [146], and in honeybees, smRNAi improved bee survival against parasitic mites [147]. The engineered symbionts could be detected more than 250 days after initial uptake and were horizontally transmitted [146,147]. In contrast, application of smRNAi to aphids using *E. coli* and the native symbiont, *Serratia symbiotica* CWBI-2.3T, was unable to induce reproducible aphid phenotypes or even gene knockdown [148]. This discrepancy could be partially attributed to selection of an appropriate symbiont. Genetically tractable laboratory strains like *E. coli* are foreign to the host, which can induce immune responses and reduce colonization of these transgenic bacteria. Other important caveats are the need for bacterial RNase III mutants, which promote stable dsRNA production, and localization of the symbiont within the host as how dsRNA is delivered by the symbiont during smRNAi remains unknown [148].

A similar but parallel approach for insect control termed pathogen-mediated RNAi has also emerged. Building upon their discovery of cross-kingdom RNAi between *B. bassiana* and

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mosquito [34], Cui et al. engineered *B. bassiana* to produce immunosuppressive miRNAs against *A. aegypti* and *Galleria mellonella* [149]. Topical application of transgenic *B. bassiana* expressing *A. aegypti* miR-8 and miR-375, negative regulators of the Toll immune signalling pathway, resulted in a 20–30% reduction in survival time for insecticide-resistant *A. aegypti* and *G. mellonella* larvae [149]. Importantly, miR-8 and miR-375 are conserved in several agricultural insect pests so this technology can be easily translated for control of other organisms. While the use of bacterial-based systems is relatively new compared to topical application of exogenous dsRNA, there is immense promise in this technology.

Conclusions and future directions

Nanotechnology is becoming increasingly popular in modern agriculture as a tool to address major food security challenges but has only recently been leveraged with powerful RNAi-based strategies such as SIGS to control and combat plant pests and pathogens. Specifically, the use of nanocarriers in SIGS has alleviated previous limitations with RNA uptake and stability in the environment and after ingestion (Figure 2). Many hosts and interacting microbes actively exchange sRNAs for reciprocal gene silencing, but these sRNAs must be protected from degradation to remain intact and functional. As characterized across different kingdoms of life, the use of EVs is a prevalent strategy for RNA transport and protection, and considerations of these mechanisms of native host RNA trafficking are crucial in guiding the design of effective and robust SIGS applications.

The use of nanocarriers for RNAi-based control will greatly improve the efficacy of SIGS; however, before this technology can be wholly adopted, studies must be performed examining the lifespan and fate of nanocarriers in the environment, the impact on non-target organisms, and the potential transfer to other plant hosts. A study on the rice-hopper-spider food chain found that ingestion of treated plant tissues or guttation droplets was sufficient to trigger both targeted and off-target RNAi in consumer hoppers and predator spiders [87]. Some reports have made headway in including non-target organisms when testing new dsRNA constructs or dsRNA-nanoparticle complexes, and this practice should be incorporated into future SIGS studies [81,112,113]. Modelling the fate of nanocarrier compounds after degradation and their interactions with soil and water will also complement these efforts [150,151].

Encapsulation of RNA in nanocarriers provides extensive protection from environmental conditions and nuclease degradation. Many of these nanocarriers further promote cellular uptake in target organisms by facilitating endosomal escape which significantly improves RNAi efficiency. Importantly, recent studies have generated not only preventative plant protection using RNA-nanocarrier complexes but also curative treatment of ongoing, systemic viral infections. It will be interesting to see if curative protection against microbial pathogens can be achieved. Material features including a positive zeta potential, small size, and hydrophilicity have been identified as key parameters that can be tuned to optimize RNA delivery. Thus, the development of novel and effective SIGS approaches can be accomplished by complementing these nanocarrier advances with improvements in dsRNA design and target gene selection. The costs of these technologies, both financial and environmental should continue to be considered. As well, additional work is needed

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to understand the environmental fate of dsRNA and nanocarriers during and after plant protection. Overall, the current interest and emphasis on nanocarrier design strongly supports the potential of nanotechnology to propel SIGS to be a comprehensive plant protection system against multiple pests and pathogens.

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Data availability

No data was used for the research described in the article.

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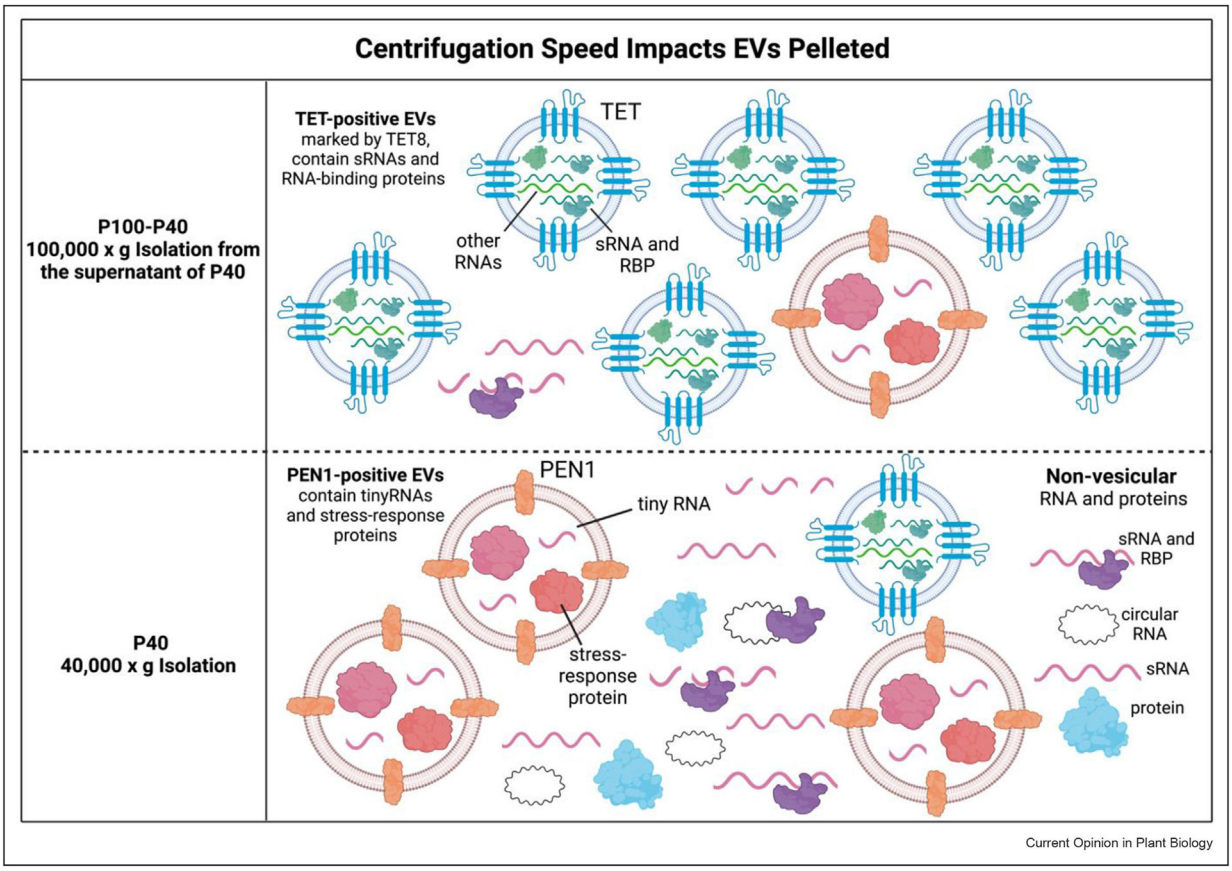

Figure 1. Centrifugation speed impacts the EV subtype that is isolated from plants.

Different ultracentrifugation speeds differentially pellet distinct plant EV subtypes. In *Arabidopsis*, 40,000×*g* collects the P40 fraction which is enriched in PEN1-positive EVs, extravesicular RNAs, and RNA-protein complexes, and misses a large portion of TET-positive EVs [51,53,54]. PEN1-positive EVs are enriched in tiny RNAs and stress-response proteins [53]. Centrifugation at 100,000×*g* (P100) is necessary to pellet plant exosomes such as TET-positive EVs [51]. The supernatant of the P40 fraction can be centrifuged at 100,000×*g* to collect TET-positive EVs in the P100–P40 fraction [51]. TET-positive EVs are enriched in sRNAs involved in cross-kingdom RNAi and RNA-binding proteins [38].

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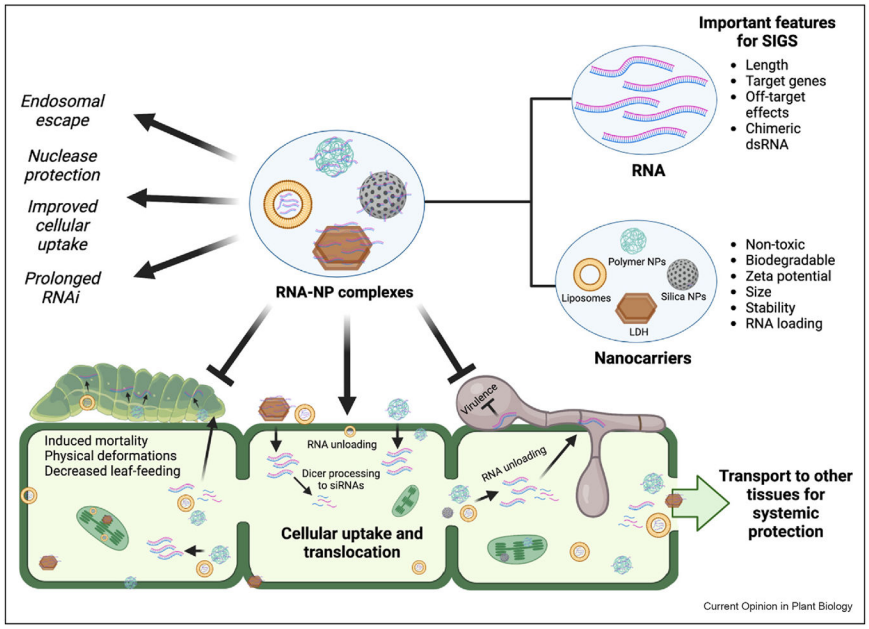

Figure 2. RNA-nanoparticle complexes provide enhanced RNA stability and uptake, leading to greater efficiency in SIGS-based crop protection strategies.

This schematic illustrates how RNA can be encapsulated in various nanocarriers to form RNA-nanoparticle complexes. Important features for dsRNA and nanocarrier design that could improve SIGS efficacy are highlighted along the side. Use of RNA-nanoparticle complexes in SIGS results in improved and prolonged gene silencing relative to naked dsRNA due to better endosomal escape, nuclease protection, and cellular uptake. RNA-nanoparticle complexes can be topically applied to plant surfaces where they are directly taken up by pests and pathogens or internalized by plant cells. After internalization, RNA is released from RNA-nanoparticle complexes and taken up by plants or pests/pathogens, translocated, and/or processed to siRNAs. RNA-nanoparticle complexes can also be directly transported to other plant tissues to provide systemic protection.