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*Review*

# RNAi Crop Protection Advances

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**Abstract:** RNAi technology is a versatile, effective, safe, and eco-friendly alternative for crop protection. There is plenty of evidence of its use through Host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS) techniques to control viruses, bacteria, fungi, insects, and nematodes. As for SIGS, its most significant challenge is achieving stability and avoiding premature degradation of RNAi in the environment or during its absorption in the target organism. One alternative is the encapsulation in liposomes, virus-like particles, polyplex nanoparticles, and bio-clay, which can be obtained through the recombinant production of RNAi in vectors, transgenesis, and micro/nanoencapsulation. The materials must be safe, biodegradable, and stable in multiple chemical environments favoring the controlled release of RNAi. Most of the current research of encapsulated RNAi focuses primarily on oral delivery to control insects by silencing essential genes. The regulation of RNAi technology focuses on risk assessment from different approaches; however, this technology has positive characteristics for its use in agriculture from the economic, environmental, and human health implications. The emergence of alternatives combining RNAi gene silencing with the induction of resistance in crops by elicitation and metabolic control is expected, as well as multiple silencing and biotechnological optimization of its large-scale production.

**Keywords:** RNAi; dsRNA; silencing, encapsulation, liposomes, virus-like particles, polyplex nanoparticles, bio-clay, regulatory.

## 1. Introduction

The world moves towards a more sustainable crop production system that urges specific and efficient tools to battle plant pathogens. RNAi can be used for such purposes. The molecule is used by nature, degrades quickly, can disrupt the pathogen at a genetic specific level, and can complement the current agronomic crop protection practices used for organic, conventional, ecological, or technological production[1]. The reader may be familiar with the concept of DNA and genes located at the nucleus of eukaryote cells, containing the instructions to elaborate organic molecules, mainly proteins. RNA messenger works as an intermediator, carrying the nucleus's message to the cytoplasm to be read by the ribosomes to ensemble the protein. RNAi eukaryotic machinery is a complex system for virus defense and gene expression control, named Post Transcriptional Gene Silencing (PTGS). The system can be triggered by external specific dsRNA resulting in its RNA messenger block before it gets to the ribosome leaving the organism, such as a pathogen, disarmed[2]. The delivery of external dsRNA to disarm the expression system was proven to be natural and bi-directional from plant to pathogen and vice versa cross-kingdom communication[3–8].

Consequently, RNAi represents an opportunity to emulated or improve the natural plant pathogen control system by providing well-designed external dsRNA[9]. Here we aimed to present advantages in crop protection mediated by RNAi. There are two RNAi plant-based technologies Host-induced gene silencing (HIGS) used since the 90s and

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spray-induced gene silencing (SIGS). Both can provide sustainable solutions to control pathogens, such as insects, viruses, and fungi. We will focus on SIGS because it is becoming an emerging affordable option with a cost reduction of about 0.5–1 USD per gram[10]; the small amount needed of dsRNA that seems to be near 2-10 grams per hectare; its safety; and fast environmental degradation[11–13]. When dsRNA is applied externally in plants, the plant cells can take it and use it directly to tackle the pathogen through secreted vesicles containing the RNA at the site of infection and plasmodesmata[14–16]. The sprayed RNA amount may vary depending on the target species' sensitivity to RNAi, the capacity for triggering the defense system, and the efficient delivery method. Other challenges for this technology are the need for science-based risk assessment procedures for topical RNAi applications within existing plant protection products legislation; the regulatory aproaches[12,17]; the strategy to use more than one target sequence to avoid resistance uptake method[18].

## 2. Potential targets

The potential targets of RNAi can be viruses, fungi, bacteria, nematodes, and endogenous genes. We describe next a general table containing several targets to demonstrate that the technology is flexible enough to start exploring other plagues, broader reviews exist in case of interest by the reader[19]

**Table 1.** Potential targets for spray-induced gene silencing (SIGS) in plants.

| Target | Experimental evidence | Target genes | Reference |
|---|---|---|---|
| Virus | dsRNA+clay resulted in BCMV virus resistance for 20d | Nib and CP genes of BCMV | 20 |
| | TMV Tobacco virus resistance for 7-20d | CP,P126,RP of TMV | 21,22 |
| Fungi | Inhibits *Botrytis cinerea* disease | DCL1, DCL2 of *Botrytis cinerea* | 23 |
| | Efficiently inhibited *Fusarium graminearum* | CYP51A, CYP51B, CYP51C of *F. graminearum* | 24 |
| | *Sclerotinia sclerotiorum/ Botrytis cinerea* | mRNA splicing, ribosome biogenesis, protein disulphide oxidoreductase, peroxisomal protein | 23 |
| | *Fusarium asiaticum, Botrytis cinerea Magnaporthe oryzae Colletotrichum truncatum,* | *β2-tubulin* | 25 |
| | *Fusarium oxysporum f. sp. cubense and Mycosphaerella fijiensi, Fusarium* | adenylate cyclase, DNA polymerase alpha subunit/delta subunit/ CYP51 | 26,27 |
| Nematodes | *Caenorhabditis elegans, Radopholus similis, Meloidogyne artiellia, Meloidogyne incognita, Globodera pallida* | Several genes | 24 |
| Insects | Coleopterans are highly sensitive, Hemiptera, Orthoptera, Diptera, Hymenoptera, and Lepidoptera have different responses. | Several genes | 25 |
| Endogenous plant genes | Arabidopsis, Tobacco, poplar, rice | Transgenes/CHS/EPSPS/STM/WER/ MYB1/ WRKY23 | 30 |

BCMV:potyvirus *Bean Common Mosaic Virus*, TMV: *Tobacco Mosaic Virus*

## 3. Encapsulation technology to improve efficiency

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The use of encapsulation technology has improved the effectiveness of gene silencing by designed RNAi. It confers protection and stability to the dsRNA preventing it from undergoing enzymatic or pH degradation while it is transported to the target cells where the release of the dsRNA and its subsequent transformation into siRNA is required [31–33]. The development of the encapsulation system is related to the target organism, the type of RNAi to be delivered, and its uptake mechanism. According to this review, it is more common to find encapsulation of dsRNA for the control of insects by the oral route since they have an alkaline pH in the intestine and the presence of RNases in their digestive tract that would degrade naked RNAi[34]. A similar case occurs for nematodes, with the difference that the pH in their intestine is acidic[35]. Research in plants and fungi has shown that they are receptive to dsRNA and siRNA[24,36,37]. However, in insects, it has been proposed that silencing is more efficient when long dsRNA (> 50bp) is used compared to sRNA, partly related to the selectivity of its incorporation mechanism[38].

The encapsulation can be produced by engineered micro-organisms or synthetic micro/nanoparticles[39] using different materials like proteins[40], biopolymers[41], clays[31] or lipids[42]. Using these materials confers valuable properties for integrating siRNAs in target cells; for instance, capsid proteins that are already recognized by the target organism facilitate the penetration of dsRNAs into their cells, taking advantage of natural infection mechanisms[43]. There are multiple reports of engineered encapsulation systems that transport and protect RNAi, becoming suitable crop protection applications (Table 2).

**Table 2.** Summary of crop protection application using encapsulation system for RNAi delivery.

| Encapsulation system | Potential crop protection application | Strategy | Reference |
|---|---|---|---|
| Guanylated 2-(aminoethyl) methacrylate (AEMA)/dsRNA polyplex nanoparticles. | Insecticide induces decreased feeding in Lepidopteran larvae (*Spodoptera exigua*); then, promoting weight loss, developmental halt, and mortality. | Increases the RNAi efficiency in targeting the essential gene *chitin synthase B* (*ChSB*), while preventing degradation of dsRNA in the alkaline gut of insects and enhancing its cellular uptake in the midgut cells. | 34 |
| poly-[N-(3-guanidinopropyl) methacrylamide] (pGPMA)/dsRNA interpolyelectrolyte nanocomplex. | Ingestion insecticide regulates gene silencing in Lepidopteran larvae (*Spodoptera frugiperda*), increasing mortality from starvation and growth stunting. | Increased internalization and protection of dsRNA in insect cells, decreasing the accumulation of target mRNA due to the knockdown of genes related to vital functions such as nutrient absorption (*sfVATPase*), intracellular transport (*sfKIF*), and cell division (*sfCDC27*). | 41 |
| Chitosan/dsRNA polyplex nanoparticles | Nematicide can homogeneously enter the nematode's body (*Caenorhabditis elegans*) through non-canonical endocytotic pathways and attack specific genes. The combined effect decreases | Increases the RNAi efficiency of gene knockdown throughout the whole body of the nematode by introducing intact dsRNA through the Clathrin-mediated endocytosis pathway, which is different from the canonical pathway (sid-1 and sid-2) in the study model. Furthermore, chitosan was shown to effectively decrease the myosin gene expression, which is critical for the growth and reproduction of the model nematode. | 35 |

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| | the development of the nematode by the action of the chitosan vehicle. | | |
| Chitosan/dsRNA polyplex nanoparticles | Insecticide against Lepidopteran larvae (*Spodoptera frugiperda*) acts on genes related to the apoptosis pathway, inducing growth impairment and larval mortality. | Improve RNAi efficiency through the protection of dsRNA from degradation by intracellular and intercellular RNases. It also reduced the accumulation of dsRNA in the endosome while favoring its transport to the cytoplasm, where the formation of siRNAs is promoted, producing knockdown of apoptosis-related genes (*iap*). | 32 |
| Layered double hydroxide (LDH) clay nanosheets/dsRNA | Develop a topical product that induces viral resistance in plants (against PMMoV and CMV) using dsRNA absorption technology in clay nanosheets (Bio-Clay). | Increased persistence of the topical treatment due to the strong adhesion of the dsRNA in the vehicle (LDH) and of this with the leaves. It also allows the controlled release of the biomolecule and confers protection against environmental degradation while favoring the internalization of dsRNA in the plant. | 31 |
| Lipofectamine 2000 liposomes/dsRNA. | Insecticide against Diptera of the genus Drosophila (*D. melanogaster*, *D. sechellia*, *D. yakuba*, and *D. pseudoobscura*) acting by ingestion. It attacks essential genes of development through knockdown management. | Promotion of dsRNA internalization in insects through encapsulation protection, increasing silencing efficiency by promoting more significant RNAi accumulation in larvae. Knockdown of the genes of the *VATPase* (gut lumen pH stabilizer associated with nutrient uptake) and *gTub23C* (mitosis-related g-tubulin protein, essential for microtubule organization). | 44 |
| Lipofectamine 2000 liposomes/dsRNA. | Specific insecticide against larvae and adults of *Drosophila suzukii* combining synergic effect of multiple gene knockdown. Oral administration route. | It facilitates the uptake in the insect´s gut. It causes significant mortality in larvae and adults by the reduction in transcript levels of essential genes *rps13* (housekeeping), *alpha COP* (coatomer subunit for trans-organelles transport), and *vha26* (subunit of the vacuolar ATPase). The synergistic action of knockdown of the *rps13* and *alpha COP* genes significantly increases mortality in the insect. | 42 |
| Liposomes/dsRNA | Oral insecticide for the control of nymphs of *Euschistus heros* (hemiptera: pentatomidae), which is | Protection of dsRNA against degradation promoted by the ribonuclease action of insect saliva. Enhanced silencing activity of target genes *vATPase*A (V-type proton ATPase catalytic subunit A) and *act-2* (muscle actin). | 45 |

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|---|---|---|---|
| | one of the main soybean pests in the field. | | |
| Recombinant Flock House Virus FHV/dsRNA | Recombinant insecticide based on a viral vehicle transporting dsRNA silencers of essential genes in *Drosophila melanogaster*. For potential massive application in other species susceptible to FHV infection. | Use of the insect cell machinery to assemble infective recombinant FHV virions that carry target sequences for the production of dsRNA when replicating in cells. Thus, virions protect the sequences responsible for silencing the *rps13* (housekeeping), *alpha COP* (coatomer subunit for trans-organelle transport), and *vha26* (subunit of the vacuolar ATPase) genes while at the same time favoring dispersal in insects. It simulates natural viral infection. | 43 |
| Virus Like Particles (VLP)/dsRNA | Oral insecticide for the control of ants of several genera (*Solenopsis invicta* (fire ants), *Camponotus pennsylvanicus* and *Camponotus floridanus* (carpenter ants), *Linepithema humile* (Argentine ants), *Tapinoma sessile* (odorous ants), *Tetramorium caespitum* (pavementom ants), and Monstrous ants) pharaonis (pharaoh ants); inducing the silencing of physiological genes required for the survival of the colony. | Recombinant production in *E. colli*, which through specific plasmids manufacture capsid proteins of bacteriophages Qß and MS2 and inducible RNAi precursor sequences. The packaging of the dsRNAs in the VLPs protects them from degradation by non-specific environmental organisms and the intestinal RNases of the target organism. It also favors its absorption by lining the gut cells. The silenced genes are related to the viability of the colony, for example, the induction of sterility and individual mortality. VLP carrying dsRNA is sprayed on the ground for spot application or incorporated into the bait. Target genes included *VgR* (vitellogenin receptor protein), *TVXI* (telomerase variant XI protein), *PBAN* (pheromone biosynthesis activating neuropeptide), *PBANR* (pheromone biosynthetic activating neuropeptide receptor), *WLS* (wntless protein), *MEGF10* (multiple epidermal growth factor-like domain proteins 10), *CHCP* (clatherin heavy chain protein), *CDC7* (cell division cycle 7-related protein), *Cep89* (centrosomal protein 89 kdal), *PSMBl* (beta subunit of the type-1 proteosome), *A5C* (actin 5C protein), *ATPSD* (ATP synthase delta subunit); as well others related with anamorsin, beta actin, and Csp9 proteins | 33 WO2017/136353Al for APSE RNA Containers (ARCs) |
| Ribonucleoprotein particle (RNP)/dsRNA | Insecticide for control of the Cotton boll weevil (*Anthonomus grandis*) adults. | Developing a protection and stability system for dsRNA avoids degradation by nucleases in the insect´s gut and favors rapid cellular incorporation. The above is based on a chimeric protein PTD-DRBD (peptide transduction domain — dsRNA binding domain) combined with dsRNA. This type of resulting protein is known as cell-penetrating peptides (CPP). | 40 |

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According to the data available in the references, we identified four principal encapsulation systems: the formation of liposomes, virus-like particles, polyplex nanoparticles, and bio-clay. As mentioned in table 2, these systems coincide in the biodegradability of the materials, the ability to improve the stability of RNAi, and its synergistic effect to induce control. Figure 1 presents a diagram of the most reported systems.

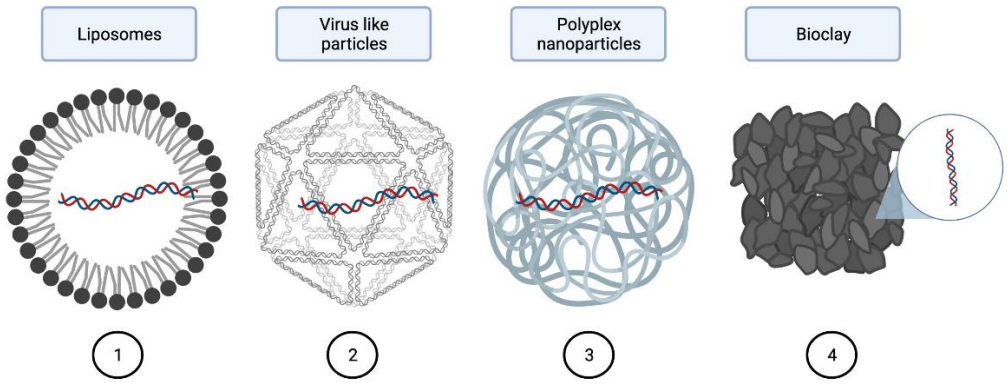


**Figure 1.** Primary encapsulation system for RNAi delivery.

## 4. Regulatory approaches

The time and cost associated with obtaining the data for a registry of a Biomolecule like dsRNA can be low compared with a conventional pesticide of 4 versus 12 years and 3-7million USD versus 280USD[46]. An important consideration is that dsRNA generally have low environmental persistence in soil, sediment, and water[47,48]. The biomolecule shows a record of safe consumption of short and long RNAs in the diet from food and lacks oral immunostimulation[11]. Another positive input when regulating this technology is the technical discussion that has resulted in the last decade where USEPA and OECD (Organisation for Economic Co-operation and Development) propose using and adapting the existing plant protection products norms and procedures as described next. The United States Environmental Protection Agency (EPA) has analyzed addressing this technology based on problem formulation for Human Health and Ecological Risk Assessment. At the same time, OECD proposes using risk assessment to evaluate the toxicity profile and exposure of the molecule by adapting the current regulatory framework for small molecule agrochemicals as a general framework for dsRNA-based agricultural products; and proposes taking into consideration the experience with the review of dsRNA-based GE crops. EFSA literature review on GM plants is a document to be taken into consideration as well.

**Table 3.** Regulatory approaches

| Regulatory Agency | Proposal | Reference |
|---|---|---|
| EPA | Propose using Problem Formulation-Risk assessment | 49 |
| European Food Safety Authority (EFSA) | Do not directly address the spray products, but a literature review focus on RNAi-based GM plants and Risk Assessment | 50 |

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|---|---|---|
| OECD | Propose using risk assessment to evaluate the toxicity profile and exposure, by using the current regulatory framework for small molecule agrochemicals as a general framework for dsRNA-based agricultural products. Proposes using the experience with the review of dsRNA-based GE crops | 11 |

## 5. Conclusions and future perspectives

RNAi technology is a powerful and versatile alternative for pest and disease control in crops. Its use in the agricultural field extends to viruses, bacteria, fungi, insects, nematodes, and plants. It grows steadily with other complementary technologies such as the recombinant production of RNAi in vectors, transgenesis, and micro/nanoencapsulation of candidate si/dsRNA. The main issue avoiding its adoption in the past was the cost of production and stability. The cost of production is getting lower with the development of new technologies, while stability encapsulation strategies provide a solution to avoid degradation.

Encapsulation of RNAi in liposomes, virus-like particles, polyplex nanoparticles, and bio-clay have gained relevance in the last decade because they confer protection against degradation. Reducing this degradation has been a challenge for the evolution of this technology. This degradation occurs on naked dsRNAs because of environmental exposure or the action of enzymes and the pH level of the target organism. Encapsulation also provides stability to the dsRNA and sometimes favors cell uptake. Some of the materials used for encapsulation provide additive effects on pest control; however, most of them are innocuous, biodegradable, and stable in multiple chemical environments favoring the controlled release of RNAi. Our review found multiple reports of this technology applied mainly for the control of insects, where the predominant administration mechanism is the oral route using spray-induced gene silencing (SIGS) or the application of encapsulation on baits.

Candidate genes for targeted silencing coincide in essential genes related to enzymes involved in cell division (e.g., CDC27, gTub23C, TVXl, Cep89), cell transport (e.g., KIF, alpha COP), structure formation (e.g., ChSB, act- 2, MEGF10, A5C), and ionic balance and nutrient absorption (e.g., V-ATPase, vha26,); then, producing mortality in the target species.

Current regulations on products developed with RNAi technology focus on assessing their risk from different approaches. However, based on the characteristics of these biomolecules and their proven safety in non-target organisms, a favorable position is predicted for the use of this technology in agriculture, where the will to regulate is optimistic regarding the economic and environmental advantages and its low risks associated with human health. The regulatory landscape can allow the safe adoption of this technology with the current decision-making based on risk assessment. However, a harmonized approach will be needed to enable adoption and avoid trade disruptions soon.

Together with the positive evolution in regulatory, the emergence of more interdisciplinary alternatives that combine gene silencing by RNAi is also expected. For instance, the induction of resistance in crops by elicitation and metabolic control methods, using the strengths of both. Following this approach, we are developing a technology that uses elicitor nanoparticles made of natural polymers to induce defense in the plants, which will also carry a double control mechanism based on RNAi to unblock inhibitors of systemic defense against systemic defense pathogenic species of the genus *Fusarium*. There is also interest in the scientific community to produce multiple knockdowns that protect systemically against a consortium of pathogens under the same application. Another challenge for this technology is to keep reducing production costs, for which biotechnology is emerging as one of the main allies to produce profitably and on a large scale.

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