doi:10.20944/preprints202110.0060.v2

Review

RNAi Crop Protection Advances

Alejandro Hernández-Soto[1]*, Randall Chacón-Cerdas[2]

[1] Costa Rica Institute of Technology, DOCINADE, Biology School, Biotechnology Research Center. Costa Rica., P.O Box 159-7050 Cartago, Costa Rica; *alhernandez@tec.ac.cr* (https://orcid.org/0000-0001-9435-5117)
[2] Costa Rica Institute of Technology, Biology School, Biotechnology Research Center. Costa Rica., P.O Box 159-7050 Cartago, Costa Rica; *rchacon@tec.ac.cr* (https://orcid.org/0000-0002-5364-4649)
* Correspondence: *alhernandez@tec.ac.cr*

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 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. How it works?

The system was discovered in 1998 when the nematode *Caenorhabditis elegans* exposed to a double-stranded RNA injection resulted in gene silencing[19]. The finding received a Novel Prize award in Physiology and Medicine in 2006. The cascade of reactions where the introduced dsRNA ends up knocking out other RNAs is fascinating and complex. The native system is the microRNA and works as a post-transcriptional gene silencing (PTGS), orchestrating internal gene expression when needed. It starts with genomic codifying sequences transcribed by an RNApolII, producing the first double-stranded RNAs pri-miRNA processed in the nucleus by several enzymes such as Drosha and DGCR8, into a pre-miRNA. The molecule is exported to the cytoplasm by Exportin-5 and, with the help of several enzymes (Helicase, Dicer, TRBP, and Agr-2 TNRC6), end up in a piece of activated machinery "RISC complex" capable of repressing, destroying, or even inactivating genes at the genetic level based on the resulting RNA single-stranded template[1,20–22].

What is extraordinary is how the RISC complex evolved to become a defense system capable of using external dsRNA sequences, known as short interference RNA system (siRNA). The defense system usually works for viral defense. The scientist took advantage of it when adding external dsRNA to study gene expression in a model organism *C. elegans* [19,23]. Long dsRNA given to the nematode triggers the system. The first enzyme, Dicer, recognizes the molecule and cuts it into smaller fragments of 20-40nts. RISC complex can then separate the strand and use it to silence a complementary messenger RNA. Fungi and Plants produce their dsRNA to disarm each other in a cross-kingdom communication fight. Even more fascinating is that plants can take external dsRNA from cell to cell and deliver vesicles with the RNA against fungi[7,14]. Figure 1 shows a diagram of spray-induced gene silencing (SIGS) mechanism mentioned above.

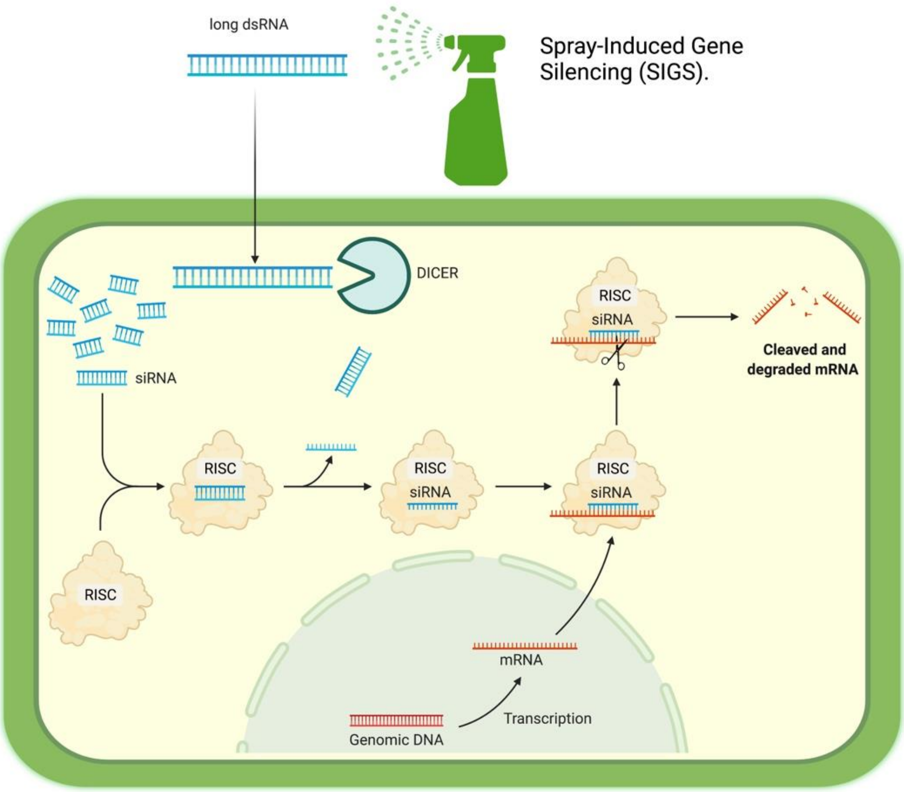

Figure 1. Spray-induced gene silencing (SIGS) mechanism.

3. 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[24]. Out these possibilities we would like to remark the potential of using SIGS (spray-induced gene silencing) to allow a new generation of crop protection specific based products.

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	25
	TMV Tobacco virus resistance for 7-20d	CP,P126,RP of TMV	26,27
Fungi	Inhibits *Botrytis cinerea* disease	DCL1, DCL2 of *Botrytis cinerea*	28
	Efficiently inhibited *Fusarium graminearum*	CYP51A, CYP51B, CYP51C of *F. graminearum*	29
	Sclerotinia sclerotiorum/ Botrytis cinerea	mRNA splicing, ribosome biogenesis, protein disulphide oxidoreductase, peroxisomal protein	28
	Fusarium asiaticum, Botrytis cinerea Magnaporthe oryzae Colletotrichum truncatum,	*β2-tubulin*	30

	Fusarium oxysporum f. sp. cubense and Mycosphaerella fijiensi, Fusarium	adenylate cyclase, DNA polymerase alpha subunit/delta subunit/ CYP51	31,32
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	34

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

4. Encapsulation technology to improve efficiency

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 [35–37]. 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[38]. A similar case occurs for nematodes, with the difference that the pH in their intestine is acidic[23]. Research in plants and fungi has shown that they are receptive to dsRNA and siRNA[29,39,40]. 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[41].

The encapsulation can be produced by engineered micro-organisms or synthetic micro/nanoparticles[42] using different materials like proteins[43], biopolymers[44], clays[35] or lipids[45]. 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[46]. 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.	38

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*).	44
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 the development of the nematode by the action of the chitosan vehicle.	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.	23
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*).	36
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.	35
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	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).	47

	development through knockdown management.		
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.	45
Liposomes/dsRNA	Oral insecticide for the control of nymphs of *Euschistus heros* (hemiptera: pentatomidae), which is one of the main soybean pests in the field.	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).	48
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.	46
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	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), *TVXl* (telomerase variant XI protein), *PBAN* (pheromone biosynthesis activating neuropeptide), *PBANR* (pheromone biosynthetic activating neuropeptide receptor), *WLS* (wntless protein), *MEGF10* (multiple epidermal growth factor-like	37 WO2017/136353Al for APSE RNA Containers (ARCs)

	genes required for the survival of the colony.	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	
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).	43

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 2 presents a diagram of the most reported systems.

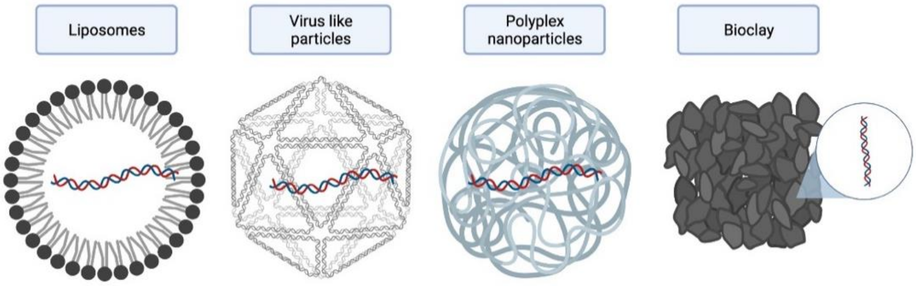

Figure 2. Primary encapsulation system for RNAi delivery.

4.1 Liposomes

Liposomes are spherical artificial vesicles synthesized from cholesterol and non-toxic natural phospholipids. They present hydrophilic and hydrophobic characteristics (amphiphilic molecules) that facilitate their interaction in multiple chemical environments[49]. The hydrophobic part consists of two fatty acid chains (10-24 carbon atoms and 0-6 double bonds in each chain) while the hydrophilic section is mainly phosphoric acid bound to a water-soluble molecule[50]. They are made up of at least one phospholipid layer. The type of lipid defines properties such as surface charge, solubility, and size of the vesicles; therefore, it is critical in the functionality of the particle. For example, unsaturated phosphatidylcholine lipids form much more permeable but less stable bilayers, while saturated phospholipids with long acyl chains form more rigid and waterproof structures. According to their size, they are classified as very small (0.025 µm) or large (2.5 µm) vesicles[49]. Multiple types of lipids are used as raw material, the most frequent in the literature being phosphatidylglycerol (PG), phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), dipalmitoylphospha-tidylcholine (DPPC), dicetylphosphat, cholesterol (CH) stearylamine, or its mixture[51].

One advantage of this type of particle is its semi-permeable character that simulates biological membranes allowing smart delivery of the compounds it carries due to its cellular penetration capacity[52]. Also, the low solubility in hydrophilic environments is an advantage when looking for a slow-release mechanism, which helps immobilize compounds and facilitate their environmental biodegradation. However, this technology also has some technical disadvantages such as a short half-life due to oxidation and hydrolysis of phospholipids, vulnerability to temperature, and others related to its cost of production [49]. The most cited synthesis methods are injection, electroformation, reverse-phase evaporation, hydration or thin-film hydration (Bangham method), microfluidic, freeze drying of double emulsions, heating membrane extrusion, detergent depletion, and supercritical fluids preparation. The challenge of the methods is to achieve stable vesicles of controlled and functional sizes[51,53].

The applications of liposomes are very versatile; for example, in the case of human and animal cells they include a vast inventory of drug delivery systems to treat multiple diseases by reducing collateral damage in non-target tissues and excessive doses[52]. In food science, they are used for the protection and controlled delivery of enzymes, vitamins, functional compounds, antibiotics, and metabolites to improve the properties of food[54]. In plants, in addition to the reports in Table 2 for RNAi delivery, they are used as carriers of CRISPR/Cas gene-editing systems, proteins, DNA, or mRNA[55]. Also, lipofection (liposome-mediated DNA delivery) in protoplasts[56], and nutrient transport /internalization are cited[57].

4.2 Virus Like-Particles (VLPs)

Virus Like-Particles (VLPs) are defined as supramolecular self-assemblies of proteins of about 10–200 nm in diameter, presenting the same or similar structure as native virions. VLPs are not infectious since they lack infective genetic material[58]. In addition to being nanocarriers, VLPs can be functionalized on the surface by ligand proteins, exhibiting additional properties like immunological and labeling reactive to changes in the chemical environment[59,60].

VLPs can be produced by heterologous expression systems such as baculovirus, bacterias, yeast, plants, and animal cells (insects, mammalians, etc.). The expression vector to be used must be selected depending on the type of the desired protein and the target organism since the system's success may depend on the post-translational modifications. According to the type of virus from which they are derived, VLPs are classified as from: single-stranded RNA positive-sense viruses, single-stranded RNA negative-sense viruses, double-1 stranded RNA viruses, single- and double-1 stranded DNA viruses. Also, VLPs can be produced by crude protein extracts (Cell-free system), mainly when the heterologous expression implies the production of toxic compounds for the vector[61]. Furthermore, the expression systems have different yields, being generally more efficient in viral and bacterial vectors. The VLP surface conjugation can be covalent and non-covalent type; the first option is preferred to functionalize with large molecules, even complete proteins. This type of conjugation is frequent through bacteriophage expression systems (MS2 and Q-beta)[58]. Regarding non-covalent conjugation, a biotinylation process is required. It implies a linking agent such as streptavidin to ensure surface fixation[62].

Some of the VLPs advantages are the self-assembly, repetitive structural stability, and resulting polydispersity systems. It improves the uptake by target cells compared to naked molecules[63,64]. Using cell recognition proteins represents lower barriers to bio interaction[46]. However, its production is not exempt from limitations related to vectors and strategic aspects in terms of system complexity, speed of expression, performance, scalability, and regulatory[59].

The main application of this technology is for prophylactic vaccines; However, their valuable properties allow them to be used as nanocarriers in drug and gene therapy, as scaffolds for bioimaging, and for the synthesis of bionanomaterials[64–66]. Regarding its uses in plants and plant cells, in addition to being vehicles for the transport of RNAi, it is used to carry the CRISPR/Cas complex as a DNA-free gene editing system[67].

4.3 Polyplex nanoparticles

The term polyplex nanoparticles refer to the encapsulation of genetic material in polymeric particles. The polymers used are polycations that electrostatically interact with negatively charged nucleic acid molecules. Thus, neutralization of charges (phosphodiester groups) and the consequent compaction in a colloidal complex occurs. It is also stabilized by hydrogen bonding interactions between the components[68]. The final properties of the polyplex depend on the physicochemical characteristics of the pristine polymers and the resulting nanoparticle features: size, surface charge, polydispersion, and hydrophilicity[69].

There is a large number of polymers for the manufacture of polyplexes, a limited list of the most used includes poly (ethyleneimine) (PEI)[69], poly-L-Lysine (PLL), poly (amidoesters) (PAE)[70], polyamidoamine (PAMAM), poly (2-dimethylaminoethyl methacrylate) (PDMAEMA)[71], polyethylenglycol (PEG), Chitosan[72], as well as copolymeric combinations such as phosphorylcholine-modified polyethyleneimine (PEI) PEGylated PEI-based, PEGylated poly (dimethylaminomethyl methacrylate) contai-ning folate, PEI-graftedα, β-poly (N-3-hydroxypropyl) -DL-aspartamide, galactose-modified trimethyl chitosan-cysteine-based, and others[73]. Theoretically, any positively charged polymer could form a polyplex, being of particular interest those biodegradable ones such as PEG, poly (glutamic acid) (PGA), poly (caprolactone) (PCL), poly (D, L-lactide-co- glycolide) (PLGA), poly (lactic acid) (PLA), N- (2-hydroxypropyl) -methacrylate copolymers (HPMA), polystyrene-maleic anhydride copolymer, and poly (amino acids) (PAAs)[73]. Polycations can also be synthesized by ring-opening polymerization controlling the resulting charge[74].

The synthesis methods of polyplexes are very diverse, defined by the raw material and the application. There are promising methodologies such as the three-dimensional hydrodynamic focusing (3D-HF) technique supported by microfluidic devices. It seeks to optimize the properties and the resulting system's effectiveness; since conventional methods can become a limitation, some of them involve an organic-aqueous interface, shear stress, harts pH and temperature levels that can damage the genetic material[70]. Another limitation is the reduced biocompatibility and biodegradability of some polymers, as well as their cytotoxicity, requiring the production of derivatives to cope with these disadvantages[75].

However, they are a widely used transport mechanism for genetic material because they manage to avoid the degradation of genetic material caused by extracellular enzymes and precisely direct the load to target cells, increasing the effectiveness of gene expression systems[70]. Nanoparticles uptake occurs by endocytosis involving interaction with cell membrane glycoproteins and the formation of the endosome, followed by its release into the cell cytoplasm and further translocation to the target organelles[68].

Its main application is as a non-viral vector for DNA transfer in gene therapy including regenerative medicine[70], AIDS, cancer, and hereditary disorders treatment[71]. It has also increased its use for transfection and editing of genomes in plants[76,77], as well as for gene silencing systems mentioned above aimed to disease and pest control[23,38,44,78,79].

4.4 Bio-clays

The Bio-clay systems are based on 10-100nm nano-clays particles able to transport selected compounds like nucleic acid. Nano-clays are stratified aluminosilicates of a single (0.7 nm thick) or double layer (1 nm thick) with variable plasticity and swelling capacity. Its chemical formula is $(Ca, Na, H)\ (Al, Mg, Fe, Zn)_2(Si, Al)_4O_{10}(OH)_2\text{-}xH_2O$. There are both natural and synthetic, and their structures consist of alternating tetrahedral SiO_2 and octahedral AlO_6 sheets with varying ratios[80,81].

Its properties depend on the nature of the atoms on the surface and the exchangeable cations between layers. Clays are negatively charged due to the substitution of the Al^{+3} or Mg^{+2} ions by Si. Consequently, the surface of the nanolayer is hydrophobic due to the Si-

O covalent bonds; however, the addition of exchangeable hydrophilic cations can change the charge to positive[80].

The most used raw materials are bentonite, hectorite, montmorillonite, kaolinite, laponite, halloysite, laponite, sepiolite, saponite, and vermiculite. They are low-cost, non-toxic, and charge versatile vehicles[81,82].

Surface functionalization is frequently performed to adapt them to specific applications. Processes such as silylation prevents the aggregation of clays through the covalent modification of the surface. For example, modified montmorillonite shows improvement in the polymer mechanical performances as well as in the water absorption, cationic exchange, and ionic retention capacities. Other clays such as sepiolite are functionalized by grafting organosilane due to many silanol groups on the surface, making them excellent heavy metal removers. Halloysite is modified mainly by covalent grafting of organosilane via condensation with the hydroxyl groups [83]. In the case of synthetic clays, such as Laponite® (phyllosilicate composed of layered synthetic silicate), it is obtained from inorganic mineral salts and is superficially modified by ion exchange or covalent linkage[83].

The applications mainly cover materials science, chemistry, physics, and biology, where they become strategic materials for developing smart nanoarchitectures. Among the most documented uses are drug delivery, environmental remediation, wastewater treatment, and food packaging[83–85]. Regarding plant biotechnology, it has also been used for the manufacture of nanocomposites to improve resistance properties in wood [86,87], vehicles for the controlled dosage of nutrients, and regulating soil properties [88–90]; being relevant to mention the transformation of desert soils into cultivable fields through liquid nano-clay[91].

5. 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[92]. An important consideration is that dsRNA generally has low environmental persistence in soil, sediment, and water[93,94]. 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 (Organization 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	95
European Food Safety	Do not directly address the spray products, but a literature review focus on RNAi-based GM plants and Risk Assessment	78

Authority (EFSA)		
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

6. 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.

Author Contributions: Conceptualization, A.H-S., R.C-C.; writing—original draft preparation, A.H-S., R.C-C.; writing—review and editing, A.H-S., R.C-C.; visualization, A.H-S., R.C-C. All authors have read and agreed to the published version of the manuscript.

Funding: This article is part of the Doctoral Thesis of the first author, Doctorado en Ciencia Naturales para el Desarrollo (DOCINADE), Instituto Tecnológico de Costa Rica (TEC), Universidad Nacional, Universidad Estatal a Distancia, Cartago, Costa Rica. Also, it is part of the Doctoral Thesis of the second author, Doctorado Académico en Ingeniería (DAI), Instituto Tecnológico de Costa Rica, Universidad de Costa Rica (Project ID 1510120, funded by Vice-presidency of Research and Extension at TEC).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

(1) Hernández-Soto, A.; Echeverría-Beirute, F.; Guzmán-Hernández, T. The RNAi as a Tool to Control Tropical Pathogens. *Agronomía Mesoamericana* **2021**, *32* (1), 326–337. https://doi.org/10.15517/am.v32i1.40896.

(2) Sneha Yogindran and Manchikatla Venkat Rajam. RNAi for Crop Improvement Sneha. In *Plant Biology and Biotechnology: Volume II: Plant Genomics and Biotechnology*; Bahadur, B., Rajam, M. V., Sahijram, L., Krishnamurthy, K. v., Eds.; 2015; Vol. II, pp 623–637. https://doi.org/10.1007/978-81-322-2283-5_31.

(3) Wang, M.; Weiberg, A.; Lin, F. M.; Thomma, B. P. H. J.; Huang, H. da; Jin, H. Bidirectional Cross-Kingdom RNAi and Fungal Uptake of External RNAs Confer Plant Protection. *Nature Plants* **2016**, *2* (10), 16151. https://doi.org/10.1038/nplants.2016.151.

(4) Cai, Q.; He, B.; Kogel, K.-H.; Jin, H. Cross-Kingdom RNA Trafficking and Environmental RNAi – Natures Blueprint for Modern Crop Protection Strategies. *Curr Opin Microbiol* **2018**, *46*, 58–64. https://doi.org/doi:10.1016/j.mib.2018.02.003.

(5) Castillo-González, C.; Zhang, X. The Trojan Horse of the Plant Kingdom. *Cell Host and Microbe* **2018**, *24* (1), 1–3. https://doi.org/10.1016/j.chom.2018.06.015.

(6) Zhao, Y.; Liang, X.; Zhou, J.-M. Small RNA Trafficking at the Forefront of Plant–Pathogen Interactions. *F1000Research* **2018**, *7* (0), 1633. https://doi.org/10.12688/f1000research.15761.1.

(7) Cai, Q.; Qiao, L.; Wang, M.; He, B.; Lin, F.-M.; Palmquist, J.; Jin, H. Plants Send Small RNAs in Extracellular Vesicles to Fungal Pathogen to Silence Virulence Genes. *Science* **2018**, *360* (June), 1126–1129. https://doi.org/10.1126/science.aar4142.

---

(8) Weiberg, A.; Wang, M.; Lin, F.-M.; Zhao, H.; Zhang, Z.; Kaloshian, I.; Huang, H.-D.; Jin, H.; Khaloshian, I.; Huang, H.-D.; Jin, H. Fungal Small RNAs Suppress Plant Immunity by Hijacking Host. *Science (New York, N.Y.)* **2014**, *342* (6154), 118–123. https://doi.org/10.1126/science.1239705.Fungal.

(9) Giudice, G.; Moffa, L.; Varotto, S.; Cardone, M. F.; Bergamini, C.; de Lorenzis, G.; Velasco, R.; Nerva, L.; Chitarra, W. Novel and Emerging Biotechnological Crop Protection Approaches. *Plant Biotechnology Journal* **2021**, 1–16. https://doi.org/10.1111/pbi.13605.

(10) Taning, C. N. T.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Mezzetti, B.; Sabbadini, S.; Sorteberg, H. G.; Sweet, J.; Ventura, V.; Smagghe, G. RNA-Based Biocontrol Compounds: Current Status and Perspectives to Reach the Market. *Pest Management Science* **2020**, *76* (3), 841–845. https://doi.org/10.1002/ps.5686.

(11) Rodrigues, T. B.; Petrick, J. S. Safety Considerations for Humans and Other Vertebrates Regarding Agricultural Uses of Externally Applied RNA Molecules. *Frontiers in Plant Science* **2020**, *11* (April), 1–12. https://doi.org/10.3389/fpls.2020.00407.

(12) Zotti, M.; dos Santos, E. A.; Cagliari, D.; Christiaens, O.; Taning, C. N. T.; Smagghe, G. RNA Interference Technology in Crop Protection against Arthropod Pests, Pathogens and Nematodes. *Pest Management Science*. 2018, pp 1239–1250. https://doi.org/10.1002/ps.4813.

(13) Mezzetti, B.; Smagghe, G.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Kostov, K.; Sabbadini, S.; Opsahl-Sorteberg, H.-G.; Ventura, V.; Taning, C. N. T.; Sweet, J. RNAi: What Is Its Position in Agriculture? *Journal of Pest Science* **2020**, *93* (4), 1125–1130. https://doi.org/10.1007/s10340-020-01238-2.

(14) Rutter, B. D.; Innes, R. W. Extracellular Vesicles as Key Mediators of Plant–Microbe Interactions. *Current Opinion in Plant Biology*. Elsevier Ltd 2018, pp 16–22. https://doi.org/10.1016/j.pbi.2018.01.008.

(15) Song, X. S.; Gu, K. X.; Duan, X. X.; Xiao, X. M.; Hou, Y. P.; Duan, Y. B.; Wang, J. X.; Yu, N.; Zhou, M. G. *Secondary Amplification of SiRNA Machinery Limits the Application of Spray-Induced Gene Silencing*; 2018; Vol. 19. https://doi.org/10.1111/mpp.12728.

(16) Reagan, B. C.; Ganusova, E. E.; Fernandez, J. C.; McCray, T. N.; Burch-Smith, T. M. RNA on the Move: The Plasmodesmata Perspective. *Plant Science* **2018**, *275*, 1–10. https://doi.org/10.1016/j.plantsci.2018.07.001.

(17) Mezzetti, B.; Smagghe, G.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Kostov, K.; Sabbadini, S.; Opsahl-Sorteberg, H. G.; Ventura, V.; Taning, C. N. T.; Sweet, J. RNAi: What Is Its Position in Agriculture? *Journal of Pest Science* **2020**, *93* (4), 1125–1130. https://doi.org/10.1007/s10340-020-01238-2.

(18) Wytinck, N.; Manchur, C. L.; Li, V. H.; Whyard, S.; Belmonte, M. F. DsRNA Uptake in Plant Pests and Pathogens: Insights into RNAi-Based Insect and Fungal Control Technology. *Plants* **2020**, *9* (12), 1–17. https://doi.org/10.3390/plants9121780.

(19) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and Specific Genetic Interference by Double-Stranded RNA in Caenorhabditis Elegans. *Nature* **1998**, *391* (6669), 806–811. https://doi.org/10.1038/35888.

(20) Wilson, R. C.; Doudna, J. A. Molecular Mechanisms of RNA Interference. *Annual Review of Biophysics* **2013**, *42* (1), 217–239. https://doi.org/10.1146/annurev-biophys-083012-130404.

(21) Axtell, M. J. Classification and Comparison of Small RNAs from Plants. *Annual Review of Plant Biology* **2013**, *64* (1), 137–159. https://doi.org/10.1146/annurev-arplant-050312-120043.

(22) Muthamilarasan, M.; Prasad, M. Plant Innate Immunity: An Updated Insight into Defense Mechanism. *Journal of Biosciences* **2013**, *38* (2), 433–449. https://doi.org/10.1007/s12038-013-9302-2.

(23) Lichtenberg, S. S.; Tsyusko, O. v.; Palli, S. R.; Unrine, J. M. Uptake and Bioactivity of Chitosan/Double-Stranded RNA Polyplex Nanoparticles in Caenorhabditis Elegans. *Environmental Science and Technology* **2019**, *53* (7), 3832–3840. https://doi.org/10.1021/acs.est.8b06560.

(24) Rosa, C.; Kuo, Y.-W.; Wuriyanghan, H.; Falk, B. W. RNA Interference Mechanisms and Applications in Plant Pathology. *Annual Review of Phytopathology* **2018**, *56* (1), 581–610. https://doi.org/10.1146/annurev-phyto-080417-050044.

(25) Worrall, E. A.; Bravo-Cazar, A.; Nilon, A. T.; Fletcher, S. J.; Robinson, K. E.; Carr, J. P.; Mitter, N. Exogenous Application of RNAi-Inducing Double-Stranded RNA Inhibits Aphid-Mediated Transmission of a Plant Virus. *Frontiers in Plant Science* **2019**, *10* (March). https://doi.org/10.3389/fpls.2019.00265.

(26) Konakalla, N. C.; Kaldis, A.; Berbati, M.; Masarapu, H.; Voloudakis, A. E. Exogenous Application of Double-Stranded RNA Molecules from TMV P126 and CP Genes Confers Resistance against TMV in Tobacco. *Planta* **2016**, *244* (4), 961–969. https://doi.org/10.1007/s00425-016-2567-6.

(27) Niehl, A.; Soininen, M.; Poranen, M. M.; Heinlein, M. Synthetic Biology Approach for Plant Protection Using DsRNA. *Plant Biotechnology Journal* **2018**, *16* (9), 1679–1687. https://doi.org/10.1111/pbi.12904.

(28) McLoughlin, A. G.; Wytinck, N.; Walker, P. L.; Girard, I. J.; Rashid, K. Y.; de Kievit, T.; Fernando, W. G. D.; Whyard, S.; Belmonte, M. F. Identification and Application of Exogenous DsRNA Confers Plant Protection against Sclerotinia Sclerotiorum and Botrytis Cinerea. *Scientific Reports* **2018**, *8* (1), 1–14. https://doi.org/10.1038/s41598-018-25434-4.

(29) Koch, A.; Biedenkopf, D.; Furch, A.; Weber, L.; Rossbach, O.; Abdellatef, E.; Linicus, L.; Johannsmeier, J.; Jelonek, L.; Goesmann, A.; Cardoza, V.; McMillan, J.; Mentzel, T.; Kogel, K. H. An RNAi-Based Control of Fusarium Graminearum Infections Through Spraying of Long DsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathogens* **2016**, *12* (10), 1–22. https://doi.org/10.1371/journal.ppat.1005901.

(30) Gu, K. X.; Song, X. S.; Xiao, X. M.; Duan, X. X.; Wang, J. X.; Duan, Y. B.; Hou, Y. P.; Zhou, M. G. A β 2 -Tubulin DsRNA Derived from Fusarium Asiaticum Confers Plant Resistance to Multiple Phytopathogens and Reduces Fungicide Resistance. *Pesticide Biochemistry and Physiology* **2019**, *153* (September), 36–46. https://doi.org/10.1016/j.pestbp.2018.10.005.

(31) Mumbanza, F. M.; Kiggundu, A.; Tusiime, G.; Tushemereirwe, W. K.; Niblett, C.; Bailey, A. In Vitro Antifungal Activity of Synthetic DsRNA Molecules against Two Pathogens of Banana, Fusarium Oxysporum f. Sp. Cubense and Mycosphaerella Fijiensis. *Pest Management Science* **2013**, *69* (10), 1155–1162. https://doi.org/10.1002/ps.3480.

(32) Höfle, L.; Biedenkopf, D.; Werner, B. T.; Shrestha, A.; Jelonek, L.; Koch, A. Study on the Efficiency of DsRNAs with Increasing Length in RNA-Based Silencing of the Fusarium CYP51 Genes. *RNA Biology* **2020**, *17* (4), 463–473. https://doi.org/10.1080/15476286.2019.1700033.

(33) Christiaens, O.; Whyard, S.; Vélez, A. M.; Smagghe, G. Double-Stranded RNA Technology to Control Insect Pests: Current Status and Challenges. *Frontiers in Plant Science* **2020**, *11* (April), 1–10. https://doi.org/10.3389/fpls.2020.00451.

(34) Dubrovina, A. S.; Kiselev, K. v. Exogenous RNAs for Gene Regulation and Plant Resistance. *International Journal of Molecular Sciences* **2019**, *20* (9). https://doi.org/10.3390/ijms20092282.

(35) Mitter, N.; Worrall, E. A.; Robinson, K. E.; Li, P.; Jain, R. G.; Taochy, C.; Fletcher, S. J.; Carroll, B. J.; Lu, G. Q.; Xu, Z. P. Clay Nanosheets for Topical Delivery of RNAi for Sustained Protection against Plant Viruses. *Nature Plants* **2017**, *3* (January). https://doi.org/10.1038/nplants.2016.207.

(36) Gurusamy, D.; Mogilicherla, K.; Palli, S. R. Chitosan Nanoparticles Help Double-Stranded RNA Escape from Endosomes and Improve RNA Interference in the Fall Armyworm, Spodoptera Frugiperda. *Archives of Insect Biochemistry and Physiology* **2020**, *104* (4), 1–15. https://doi.org/10.1002/arch.21677.

(37) Killmer, John; Kumar, Anil; McLaughlin, Patrick. D;Humberto, J. P. Methods and Compositions for Controlling Pests. Fiiled January 31, 2017, and issued in process., 2017.

(38) Christiaens, O.; Tardajos, M. G.; Reyna, Z. L. M.; Dash, M.; Dubruel, P.; Smagghe, G. Increased RNAi Efficacy in Spodoptera Exigua via the Formulation of DsRNA with Guanylated Polymers. *Frontiers in Physiology* **2018**, *9* (APR), 1–13. https://doi.org/10.3389/fphys.2018.00316.

(39) Wang, K.; Peng, Y.; Pu, J.; Fu, W.; Wang, J.; Han, Z. Variation in RNAi Efficacy among Insect Species Is Attributable to DsRNA Degradation in Vivo. *Insect Biochemistry and Molecular Biology* **2016**, *77*, 1–9. https://doi.org/10.1016/j.ibmb.2016.07.007.

(40) Wang, M.; Weiberg, A.; Lin, F. M.; Thomma, B. P. H. J.; Huang, H. da; Jin, H. Bidirectional Cross-Kingdom RNAi and Fungal Uptake of External RNAs Confer Plant Protection. *Nature Plants* **2016**, *2* (10), 1–10. https://doi.org/10.1038/nplants.2016.151.

(41) Ivashuta, S.; Zhang, Y.; Wiggins, B. E.; Ramaseshadri, P.; Segers, G. C.; Johnson, S.; Meyer, S. E.; Kerstetter, R. A.; McNulty, B. C.; Bolognesi, R.; Heck, G. R. Environmental RNAi in Herbivorous Insects. *Rna* **2015**, *21* (5), 840–850. https://doi.org/10.1261/rna.048116.114.

(42) Kolliopoulou, A.; Taning, C. N. T.; Smagghe, G.; Swevers, L. Viral Delivery of DsRNA for Control of Insect Agricultural Pests and Vectors of Human Disease: Prospects and Challenges. *Frontiers in Physiology* **2017**, *8* (JUN), 1–24. https://doi.org/10.3389/fphys.2017.00399.

(43) Gillet, F. X.; Garcia, R. A.; Macedo, L. L. P.; Albuquerque, E. V. S.; Silva, M. C. M.; Grossi-de-Sa, M. F. Investigating Engineered Ribonucleoprotein Particles to Improve Oral RNAi Delivery in Crop Insect Pests. *Frontiers in Physiology* **2017**, *8* (APR), 1–14. https://doi.org/10.3389/fphys.2017.00256.

(44) Parsons, K. H.; Mondal, M. H.; McCormick, C. L.; Flynt, A. S. Guanidinium-Functionalized Interpolyelectrolyte Complexes Enabling RNAi in Resistant Insect Pests. *Biomacromolecules* **2018**, *19* (4), 1111–1117. https://doi.org/10.1021/acs.biomac.7b01717.

(45) Taning, C. N. T.; Christiaens, O.; Berkvens, N.; Casteels, H.; Maes, M.; Smagghe, G. Oral RNAi to Control Drosophila Suzukii: Laboratory Testing against Larval and Adult Stages. *Journal of Pest Science* **2016**, *89* (3), 803–814. https://doi.org/10.1007/s10340-016-0736-9.

(46) Taning, C. N. T.; Christiaens, O.; Li, X. X.; Swevers, L.; Casteels, H.; Maes, M.; Smagghe, G. Engineered Flock House Virus for Targeted Gene Suppression through RNAi in Fruit Flies (Drosophila Melanogaster) in Vitro and in Vivo. *Frontiers in Physiology* **2018**, *9* (JUL), 1–13. https://doi.org/10.3389/fphys.2018.00805.

(47) Whyard, S.; Singh, A. D.; Wong, S. Ingested Double-Stranded RNAs Can Act as Species-Specific Insecticides. *Insect Biochemistry and Molecular Biology* **2009**, *39* (11), 824–832. https://doi.org/10.1016/j.ibmb.2009.09.007.

(48) Castellanos, N. L.; Smagghe, G.; Sharma, R.; Oliveira, E. E.; Christiaens, O. Liposome Encapsulation and EDTA Formulation of DsRNA Targeting Essential Genes Increase Oral RNAi-Caused Mortality in the Neotropical Stink Bug Euschistus Heros. *Pest Management Science* **2019**, *75* (2), 537–548. https://doi.org/10.1002/ps.5167.

(49) Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: Classification, Preparation, and Applications. *Nanoscale Research Letters* **2013**, *8* (1), 1. https://doi.org/10.1186/1556-276X-8-102.

(50) Yadav, D.; Sandeep, K.; Pandey, D.; Dutta, R. K. Liposomes for Drug Delivery. *Journal of Biotechnology & Biomaterials* **2017**, *07* (04). https://doi.org/10.4172/2155-952x.1000276.

(51) Maja, L.; Željko, K.; Mateja, P. Sustainable Technologies for Liposome Preparation. *The Journal of Supercritical Fluids* **2020**, *165*, 104984. https://doi.org/10.1016/j.supflu.2020.104984.

(52) Olusanya, T. O. B.; Ahmad, R. R. H.; Ibegbu, D. M.; Smith, J. R.; Elkordy, A. A. Liposomal Drug Delivery Systems and Anticancer Drugs. *Molecules* **2018**, *23* (4), 1–17. https://doi.org/10.3390/molecules23040907.

(53) Wagner, A.; Vorauer-Uhl, K. Liposome Technology for Industrial Purposes. *Journal of Drug Delivery* **2011**, *2011*, 1–9. https://doi.org/10.1155/2011/591325.

---

(54) Maherani, B.; Arab-Tehrany, E.; R. Mozafari, M.; Gaiani, C.; Linder, M. Liposomes: A Review of Manufacturing Techniques and Targeting Strategies. *Current Nanoscience* **2011**, *7* (3), 436–452. https://doi.org/10.2174/157341311795542453.

(55) Alghuthaymi, M. A.; Ahmad, A.; Khan, Z.; Khan, S. H.; Ahmed, F. K.; Faiz, S.; Nepovimova, E.; Kuča, K.; Abd-Elsalam, K. A. Exosome/Liposome-like Nanoparticles: New Carriers for Crispr Genome Editing in Plants. *International Journal of Molecular Sciences* **2021**, *22* (14). https://doi.org/10.3390/ijms22147456.

(56) Ortiz-Matamoros, M. F.; Villanueva, M. A.; Islas-Flores, T. Genetic Transformation of Cell-Walled Plant and Algae Cells: Delivering DNA through the Cell Wall. *Briefings in Functional Genomics* **2018**, *17* (1), 26–33. https://doi.org/10.1093/bfgp/elx014.

(57) Karny, A.; Zinger, A.; Kajal, A.; Shainsky-Roitman, J.; Schroeder, A. Therapeutic Nanoparticles Penetrate Leaves and Deliver Nutrients to Agricultural Crops. *Scientific Reports* **2018**, *8* (1), 1–10. https://doi.org/10.1038/s41598-018-25197-y.

(58) Zepeda-Cervantes, J.; Ramírez-Jarquín, J. O.; Vaca, L. Interaction Between Virus-Like Particles (VLPs) and Pattern Recognition Receptors (PRRs) From Dendritic Cells (DCs): Toward Better Engineering of VLPs. *Frontiers in Immunology* **2020**, *11* (June), 1–22. https://doi.org/10.3389/fimmu.2020.01100.

(59) Tagliamonte, M.; Tornesello, M. L.; Buonaguro, F. M.; Buonaguro, L. *Virus-Like Particles*; Elsevier Inc., 2017. https://doi.org/10.1016/B978-0-323-39981-4/00011-7.

(60) Biabanikhankahdani, R.; Alitheen, N. B. M.; Ho, K. L.; Tan, W. S. PH-Responsive Virus-like Nanoparticles with Enhanced Tumour-Targeting Ligands for Cancer Drug Delivery. *Scientific Reports* **2016**, *6* (November), 1–13. https://doi.org/10.1038/srep37891.

(61) Zeltins, A. Construction and Characterization of Virus-like Particles: A Review. *Molecular Biotechnology* **2013**, *53* (1), 92–107. https://doi.org/10.1007/s12033-012-9598-4.

(62) Chackerian, B. Virus-like Particles: Flexible Platforms for Vaccine Development. *Expert Review of Vaccines* **2007**, *6* (3), 381–390. https://doi.org/10.1586/14760584.6.3.381.

(63) Karandikar, S.; Mirani, A.; Waybhase, V.; Patravale, V. B.; Patankar, S. *Nanovaccines for Oral Delivery-Formulation Strategies and Challenges*; Elsevier Inc., 2017. https://doi.org/10.1016/B978-0-323-47720-8.00011-0.

(64) Domingo-Espín, J.; Unzueta, U.; Saccardo, P.; Rodríguez-Carmona, E.; Corchero, J. L.; Vázquez, E.; Ferrer-Miralles, N. *Engineered Biological Entities for Drug Delivery and Gene Therapy: Protein Nanoparticles*; 2011; Vol. 104. https://doi.org/10.1016/B978-0-12-416020-0.00006-1.

(65) Le, D. T.; Müller, K. M. In Vitro Assembly of Virus-like Particles and Their Applications. *Life* **2021**, *11* (4). https://doi.org/10.3390/life11040334.

(66) Yan, D.; Wei, Y. Q.; Guo, H. C.; Sun, S. Q. The Application of Virus-like Particles as Vaccines and Biological Vehicles. *Applied Microbiology and Biotechnology* **2015**, *99* (24), 10415–10432. https://doi.org/10.1007/s00253-015-7000-8.

(67) Tsanova, T.; Stefanova, L.; Topalova, L.; Atanasov, A.; Pantchev, I. DNA-Free Gene Editing in Plants: A Brief Overview. *Biotechnology and Biotechnological Equipment* **2021**, *35* (1), 131–138. https://doi.org/10.1080/13102818.2020.1858159.

(68) Reineke, T. M.; Davis, M. E. *Nucleic Acid Delivery via Polymer Vehicles*; Elsevier B.V., 2012; Vol. 9. https://doi.org/10.1016/B978-0-444-53349-4.00239-9.

(69) Rosenkranz, A. A.; Sobolev, A. S. Polyethylenimine-Based Polyplex Nanoparticles and Features of Their Behavior in Cells and Tissues. *Russian Chemical Bulletin* **2015**, *64* (12), 2749–2755. https://doi.org/10.1007/s11172-015-1220-z.

(70) Wirth, T.; Yla-Herttuala, S. Gene Transfer Vectors (DNA Vehicles) and Their Incorporation into Biomaterials for Bone Repair. *Biomaterials for Bone Regeneration: Novel Techniques and Applications* **2014**, 374–405. https://doi.org/10.1533/9780857098104.3.374.

(71) Aggarwal, R.; Targhotra, M.; Kumar, B.; Sahoo, P. K.; Chauhan, M. K. Polyplex: A Promising Gene Delivery System. *International Journal of Pharmaceutical Sciences and Nanotechnology* **2019**, *12* (6), 4681–4686. https://doi.org/10.37285//ijpsn.2019.12.6.1.

(72) Lin, W. J.; Hsu, W. Y. Pegylation Effect of Chitosan Based Polyplex on DNA Transfection. *Carbohydrate Polymers* **2015**, *120*, 7–14. https://doi.org/10.1016/j.carbpol.2014.11.046.

(73) Vasile, C. *Polymeric Nanomaterials: Recent Developments, Properties and Medical Applications*; Elsevier Inc., 2018. https://doi.org/10.1016/B978-0-12-813932-5.00001-7.

(74) Zhang, Q. F.; Yi, W. J.; Wang, B.; Zhang, J.; Ren, L.; Chen, Q. M.; Guo, L.; Yu, X. Q. Linear Polycations by Ring-Opening Polymerization as Non-Viral Gene Delivery Vectors. *Biomaterials* **2013**, *34* (21), 5391–5401. https://doi.org/10.1016/j.biomaterials.2013.03.083.

(75) Hall, A.; Lächelt, U.; Bartek, J.; Wagner, E.; Moghimi, S. M. Polyplex Evolution: Understanding Biology, Optimizing Performance. *Molecular Therapy* **2017**, *25* (7), 1476–1490. https://doi.org/10.1016/j.ymthe.2017.01.024.

(76) Firoozi, B.; Nasser, Z.; Sofalian, O.; Sheikhzade-Mosadegh, P. Enhancement of the Transfection Efficiency of DNA into Crocus Sativus L. Cells via PEI Nanoparticles. *Journal of Integrative Agriculture* **2018**, *17* (8), 1768–1778. https://doi.org/10.1016/S2095-3119(18)61985-9.

(77) Zolghadrnasab, M.; Mousavi, A.; Farmany, A.; Arpanaei, A. Ultrasound-Mediated Gene Delivery into Suspended Plant Cells Using Polyethyleneimine-Coated Mesoporous Silica Nanoparticles. *Ultrasonics Sonochemistry* **2021**, *73*, 105507. https://doi.org/10.1016/j.ultsonch.2021.105507.

(78) Christiaens, O.; Dzhambazova, T.; Kostov, K.; Arpaia, S.; Joga, M. R.; Urru, I.; Sweet, J.; Smagghe, G. Literature Review of Baseline Information on RNAi to Support the Environmental Risk Assessment of RNAi-based GM Plants. *EFSA Supporting Publications* **2018**, *15* (5), 173p. https://doi.org/10.2903/sp.efsa.2018.en-1424.

(79) Dhandapani, R. K.; Gurusamy, D.; Howell, J. L.; Palli, S. R. Development of CS-TPP-DsRNA Nanoparticles to Enhance RNAi Efficiency in the Yellow Fever Mosquito, Aedes Aegypti. *Scientific Reports* **2019**, *9* (1), 1–11. https://doi.org/10.1038/s41598-019-45019-z.

(80) Kausar, A. *Flame Retardant Potential of Clay Nanoparticles*; INC, 2020. https://doi.org/10.1016/b978-0-12-816783-0.00007-4.

(81) Peña-Parás, L.; Sánchez-Fernández, J. A.; Vidaltamayo, R. Nanoclays for Biomedical Applications. *Handbook of Ecomaterials* **2018**, 1–19. https://doi.org/10.1007/978-3-319-48281-1_50-1.

(82) Villanova-de-Benavent, C. *Geological Overview of Clay Nanoparticles*; INC, 2020. https://doi.org/10.1016/b978-0-12-816783-0.00001-3.

(83) Massaro, M.; Cavallaro, G.; Lazzara, G.; Riela, S. *Covalently Modified Nanoclays: Synthesis, Properties and Applications*; INC, 2020. https://doi.org/10.1016/b978-0-12-816783-0.00013-x.

(84) Cavallaro, G.; Lazzara, G.; Rozhina, E.; Konnova, S.; Kryuchkova, M.; Khaertdinov, N.; Fakhrullin, R. Organic-Nanoclay Composite Materials as Removal Agents for Environmental Decontamination. *RSC Advances* **2019**, *9* (69), 40553–40564. https://doi.org/10.1039/c9ra08230a.

(85) Lisuzzo, L.; Cavallaro, G.; Parisi, F.; Riela, S.; Milioto, S.; Lazzara, G. *Colloidal Stability and Self-Assembling Behavior of Nanoclays*; INC, 2020. https://doi.org/10.1016/b978-0-12-816783-0.00004-9.

(86) Hazarika, A.; Maji, T. K. Strain Sensing Behavior and Dynamic Mechanical Properties of Carbon Nanotubes/Nanoclay Reinforced Wood Polymer Nanocomposite. *Chemical Engineering Journal* **2014**, *247*, 33–41. https://doi.org/10.1016/j.cej.2014.02.069.

---

(87) Mandal, M.; Halim, Z.; Maji, T. K. Mechanical, Moisture Absorption, Biodegradation and Physical Properties of Nanoclay-Reinforced Wood/Plant Oil Composites. *SN Applied Sciences* **2020**, *2* (2), 1–14. https://doi.org/10.1007/s42452-020-1984-0.

(88) Guo, H.; White, J. C.; Wang, Z.; Xing, B. Nano-Enabled Fertilizers to Control the Release and Use Efficiency of Nutrients. *Current Opinion in Environmental Science and Health* **2018**, *6*, 77–83. https://doi.org/10.1016/j.coesh.2018.07.009.

(89) Bernardo, M. P.; Guimarães, G. G. F.; Majaron, V. F.; Ribeiro, C. Controlled Release of Phosphate from Layered Double Hydroxide Structures: Dynamics in Soil and Application as Smart Fertilizer. *ACS Sustainable Chemistry and Engineering* **2018**, *6* (4), 5152–5161. https://doi.org/10.1021/acssuschemeng.7b04806.

(90) Songkhum, P.; Wuttikhun, T.; Chanlek, N.; Khemthong, P.; Laohhasurayotin, K. Controlled Release Studies of Boron and Zinc from Layered Double Hydroxides as the Micronutrient Hosts for Agricultural Application. *Applied Clay Science* **2018**, *152* (September), 311–322. https://doi.org/10.1016/j.clay.2017.11.028.

(91) Nourizadeh, H.; Bakhshayesh, A. Nanoclay-Based Products Across Global Markets. **2020**, No. February 2021. https://doi.org/10.22034/SAR.2020.01.

(92) Taning, C. N. T.; Arpaia, S.; Christiaens, O.; Dietz-Pfeilstetter, A.; Jones, H.; Mezzetti, B.; Sabbadini, S.; Sorteberg, H. G.; Sweet, J.; Ventura, V.; Smagghe, G. RNA-Based Biocontrol Compounds: Current Status and Perspectives to Reach the Market. *Pest Management Science* **2020**, *76* (3), 841–845. https://doi.org/10.1002/ps.5686.

(93) Parker, K. M.; Barragán Borrero, V.; van Leeuwen, D. M.; Lever, M. A.; Mateescu, B.; Sander, M. Environmental Fate of RNA Interference Pesticides: Adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environmental Science and Technology* **2019**, *53* (6), 3027–3036. https://doi.org/10.1021/acs.est.8b05576.

(94) Bachman, P.; Fischer, J.; Song, Z.; Urbanczyk-Wochniak, E.; Watson, G. Environmental Fate and Dissipation of Applied DsRNA in Soil, Aquatic Systems, and Plants. *Frontiers in Plant Science* **2020**, *11*. https://doi.org/10.3389/fpls.2020.00021.

(95) EPA. United States Environmental Protection Agency. RNAi Technology: Program Formulation for Human Health and Ecological Risk Assessment. *FIFRA Scientific Advisory Panel Meeting* **2014**, *January*, 1–77.