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REVIEW PAPER

Exogenous RNAs: promising tools for the second green revolution

Johan Rodríguez Melo, Florencia Mammarella and Federico Ariel*

Instituto de Agrobiotecnología del Litoral, CONICET, Universidad Nacional del Litoral, Colectora Ruta Nacional 168 km 0, 3000, Santa Fe, Argentina

* Correspondence: fariel@santafe-conicet.gov.ar

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Abstract

The impending need for increasing amounts of food for the world population poses enormous challenges to agriculture. Moreover, global warming has exacerbated abiotic and biotic stresses, accelerating the emergence of new pests and pathogens which threatens crop productivity. Therefore, the scientific community urgently needs to develop innovative solutions for sustainable agriculture, notably replacing synthetic pesticides by active and highly specific biomolecules for pest control. In this context, RNA-based technologies emerge as an outstanding genetically modified organism-free approach offering versatile solutions to boost productivity while conserving and harnessing the wide variety of local landraces. Here we review recent advances in the field, including RNA synthesis approaches and the development of the nanotechnology required for RNA stabilization and delivery, and we discuss the potential of RNA as the key molecule for versatile applications in the second green revolution.

Keywords: Biotechnology, crop protection, gene silencing, nanotechnology, RNA, sustainable agriculture.

Introduction

The set of research technology transfer initiatives occurring between 1950 and the late 1960s that increased agricultural production in several countries around the world was known as the Green Revolution (Hazell, 2009). In 1981, the naturalist and writer Peter Steinhart coined the term 'Second Green Revolution' to describe future widespread adoption of genetic engineering of new food crops for increased crop yield and nutrition (Steinhart, 1981). This term was also used later to refer to a combination of urban agriculture, smaller farm size, and organic agriculture with the aim of increasing resource sustainability of crop production (Dobbs, 2006). With the emergence of RNA-based technologies applied to pharma, notably for the development of mRNA vaccines, the potential of RNAs as exogenous bioactive molecules for sustainable agriculture has become of great interest.

Global warming has exacerbated abiotic and biotic stresses (Peters *et al.*, 2014), posing enormous challenges to agriculture in terms of the need for increasing amounts of food for the world population. Plant pests and pathogens significantly impair crop production, with estimated global losses ranging between 20% and 40% per year (Peters *et al.*, 2014). Over the years, the control and management of crop diseases have been based heavily on the application of a broad diversity of synthetic pesticides, including insecticides, fungicides, and herbicides—in spite of the environmental and health damage caused by the extensive use and exposure of these chemical

substances. Moreover, some of the advantages of pesticide usage such as high availability, fast action, and reliability are overshadowed by different harmful side effects such as the resurgence of the pest population, resistance development, non-target organisms, and of course potential health risks to farmers, rural populations, and consumers (Pegler *et al.*, 2019). According to the World Health Organization, nearly 1 million people are exposed to synthetic pesticides and >200 000 die every year due to intoxication with agrochemicals (Carvalho, 2017). Thus, there is an increasing motivation to develop cost-efficient, high-performing pesticides which are less harmful to the environment, while reducing people's exposure to dangerous substances. The design of novel, sustainable, and eco-friendly solutions for crop improvement and pest control is urgently needed. The design of RNA-based solutions for crop production has been explored for over a decade, and exciting results have been shared by research groups around the world. During the COVID-19 pandemic outbreak, the development of mRNA vaccines has brought this biomolecule to the center of biotechnology of the 21st century (Fang *et al.*, 2022). In addition to pharma, the potential of exogenous RNA technologies for sustainable agriculture seems limitless. In a fair analogy to vaccines, the design of highly specific, rapidly adaptable RNA-based solutions emerges as a genetically modified organism (GMO)-free approach for crop protection, capable of guaranteeing high productivity while conserving the wide variety of landraces adapted to local environmental conditions. Here we review the recent RNA-based applications and technologies for crop protection, as well as the challenges and issues derived from RNA synthesis and stabilization for efficient delivery.

RNA interference in plants: the control of endogenous gene expression and a powerful defense mechanism

The RNAi silencing phenomenon in plants involves a dsRNA precursor which induces a sequence-specific suppression of target genes (Waterhouse *et al.*, 2001) (Fig. 1). This dsRNA precursor may vary in length and origin, and can be mainly processed into two categories of active small RNAs (sRNAs) of 21–24 nt: miRNAs and siRNAs. miRNAs and siRNAs are easily recognized by the nature of their precursors. miRNAs originate from the cleavage of an imperfectly paired stem of a much larger foldback transcript and siRNAs arise from a perfectly paired dsRNA (Carthew and Sontheimer, 2009; Dalakouras *et al.*, 2020). sRNAs can participate in transcriptional and/or post-transcriptional gene silencing (TGS and PTGS, respectively). Biogenesis of miRNAs and siRNAs depend mainly upon different members from the same two families of proteins: DCL enzymes to cut them from their precursors and AGO proteins to form the RNA-induced silencing complex (RISC) (Achkar *et al.*, 2016; Singh *et al.*, 2018).

In plants, the pathway for miRNA biogenesis is unique and starts with the transcription of miRNA genes (MIRs) (Xie *et al.*, 2004). Subsequently, the miRNA pathway involves three important steps: processing, modification, and RNA-induced silencing complex (RISC) loading (Bustos-Sanmamed *et al.*, 2013; Achkar *et al.*, 2016; Singh *et al.*, 2018). First, the primary transcript of miRNAs (pri-miRNAs) which contains at least one characteristic hairpin-like (hpmiRNAs) structure is loaded into nuclear dicing bodies including DICER LIKE (DCL1) and HYPONASTIC LEAVES 1 (HYL1), among other factors (Fang and Spector, 2007; Singh *et al.*, 2022). Then, DCL1 cuts the hairpin structure on the pri-miRNA, resulting in an miRNA duplex of ~21 nt (Kurihara and Watanabe, 2004; Singh *et al.*, 2022). The methyltransferase HUA ENHANCER 1 (HEN1) adds methyl groups at the 3′ sequence of the double-stranded mature miRNA. Finally, one strand of the miRNA duplex is loaded into the Argonaute protein (AGO1) to form an miRNA-induced silencing complex (miRISC) (Baumberger and Baulcombe, 2005; Borges and Martienssen, 2015; Singh *et al.*, 2022).

On the other hand, siRNAs can be generated from dsRNA precursors of varied origin (Borges and Martienssen, 2015). Depending on the biogenesis pathway, siRNAs have been classified as: *cis*- or *trans*-natural antisense siRNAs (NATsiRNAs), formed by the annealing of two complementary and separately transcribed RNA strands (Wang and Metzlaff, 2005; Bustos-Sanmamed *et al.*, 2013); heterochromatic siRNA (hcsiRNA), derived from repetitive sequences on chromatin and transposable elements (TEs) (Bustos-Sanmamed *et al.*, 2013; Blevins *et al.*, 2015); secondary siRNAs, which are subclassified into phased siRNA (phasiRNA), *trans*-acting siRNAs (tasiRNA), and epigenetically activated siRNAs (easiRNA) (Liu and He, 2020; Wu *et al.*, 2020; Kim *et al.*, 2021; Tian *et al.*, 2021); and virus-derived siRNA (vsiRNA), which originates after virus infection (Axtell, 2013; Song *et al.*, 2019; Zhang *et al.*, 2019; Middleton *et al.*, 2021).

RNAi-based immunity in plants

RNAi in plants is one of the most conserved primary defense mechanisms against viral infections (Baulcombe, 2004). The RNAi-based machinery is triggered by dsRNAs derived from the viral infection and a subsequent amplification process. First, dsRNAs are cleaved by DCLs into 21–24 nt of siRNAs (Xie *et al.*, 2005). Next, siRNAs are loaded into the AGO proteins which mediate the repression of the target DNA (for TGS) (Zilberman *et al.*, 2003; Zheng *et al.*, 2007) or RNA (for PTGS) (Llave *et al.*, 2002; Fei *et al.*, 2021) through a sequence homology-dependent mechanism and the concerted action of core RNAi effectors.

The RNA silencing components have been widely studied over the last few years. The well-known core factors DCLs and AGOs, the RNA-dependent RNA polymerases (RdRPs),

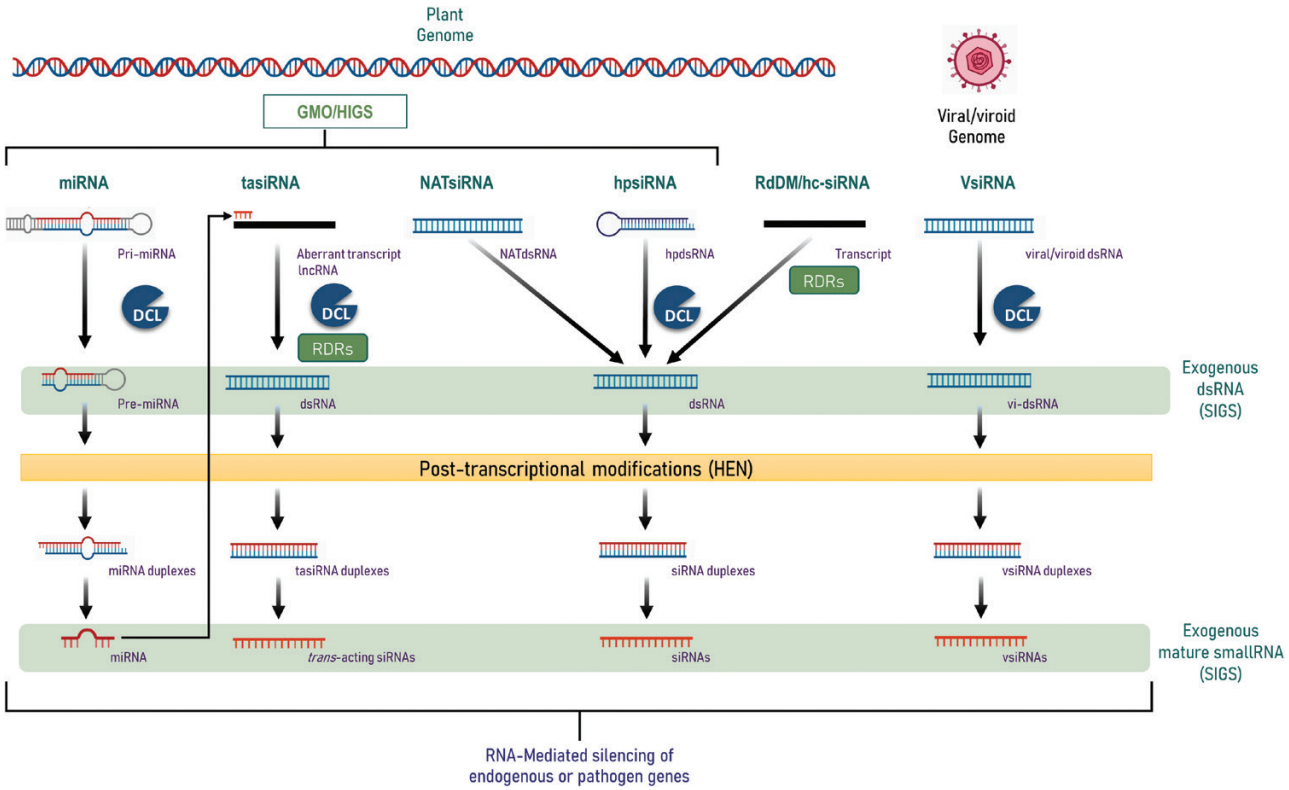

Fig. 1. Small RNA biogenesis in plants. A simplified model for the biogenesis of different classes of sRNAs. miRNA genes are transcribed by RNA polymerase II, and DCL1 processes primary RNAs into pre-miRNA, leading to the formation of miRNA duplexes. The duplex is then methylated by HEN. tasiRNA biogenesis involves an RNA polymerase II transcript which is targeted by an miRNA, then transformed into a dsRNA by RDR and processed by DCL to generate multiple mature duplexes methylated by HEN. vsiRNAs are processed by DCL, RDR, and HEN, and finally loaded on AGO to form the vsiRISC. NATsiRNAs are transcribed by RNA polymerases II/IV and are further processed by RDRs and DCLs to generate mature ds-NATsiRNA duplexes. hpsiRNA shares a similar biogenesis pathway to miRNA, although DCLs may process them, according to their secondary structure and the presence/absence of mismatches. hcsiRNA precursors are transcribed by RNA polymerase IV and serve as templates of RDR to form dsRNAs. These dsRNAs are processed into mature ds-siRNAs by specific DCLs. Exogenous application of dsRNA (spray-induced gene silencing, SIGS) or genetically modified organisms (GMO, host-induced gene silencing, HIGS) to produce dsRNA could activate the RNAi cellular mechanism in plants and/or target organisms which involves the RNAi core machinery (Meister and Tuschl, 2004; Dalakouras *et al.*, 2020). Upon cellular uptake of dsRNAs, they are processed by DCL into 20–25 nt siRNAs; one strand of mature siRNAs is incorporated into AGO protein to form the RISC. Finally, the siRNA molecules guide the RISC to scan the cytoplasm for recognition and cleavage/degradation of the complementary transcripts, thus resulting in post-transcriptional gene silencing (PTGS). DCL, RNase III enzyme DICER-LIKE (1, 2, 3, or 4, depending on the pathway); HEN1, HUA ENHANCER 1; AGO, Argonaute; RISC, RNA-induced silencing complex; RDR, RNA-dependent RNA polymerase (1, 2, or 6, depending on the pathway). Figure created with BioRender.com.

and dsRNA-binding proteins (DRBs) are fundamental components of the endogenous silencing pathways (Baulcombe, 2004; Qu *et al.*, 2008; Eamens *et al.*, 2009; Bologna and Voinnet, 2014). Also, the SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Mourrain *et al.*, 2000; Peragine *et al.*, 2004; Xie *et al.*, 2012) and HEN1 (Boutet *et al.*, 2003; Huang *et al.*, 2009) are required for RNA silencing and the generation of secondary siRNAs. Several works described a subset of other components involved in antiviral RNAi, including the lipid flippases (ALA), which play crucial roles in the biosynthesis of dsRNA precursors (Zhuo *et al.*, 2013; Guo *et al.*, 2016); the ANTIVIRAL RNAi-DEFECTIVE (AVI), an essential protein for the biogenesis of highly abundant viral siRNAs and virus-activated siRNAs (vasiRNAs) (Szittya *et al.*, 2010; Guo *et al.*, 2018); and the protein ENOR3, which functions as an enhancer of RDR6 (Gao *et al.*, 2018). More recently, Reduced Dormancy 5 (ROD5) (Liu *et al.*, 2022) was also found to function primarily in positive antiviral RNAi defense regulation. In contrast, the antiviral RNAi Regulator 1 (VIR1) was reported as a negative regulator of the antiviral RNAi response by decreasing the expression of DCL4 during the viral infection (Liu *et al.*, 2022). Taken together, these findings revealed the high complexity of the RNAi pathway in plants and the interplay with viral infections. Although further studies are needed to unravel new components and their functions in this important plant defense mechanism, a growing number of publications have demonstrated that this mechanism can be used as the basis for technological approaches enhancing pathogen-specific responses in model and crop plants (Gebremichael *et al.*, 2021; Carbonell, 2022).

Genetically modified RNAi plants triggering host-induced gene silencing

Based on the growing understanding of the RNAi silencing mechanism in plants, the scientific community succeeded in generating transgenic plants with resistance or tolerance to a wide range of pests and pathogens aiming to solve the problem of yield losses in crops worldwide. By using different stable transformation strategies, it was possible to obtain transgenic host-mediated production of dsRNA. Crop and model plants expressing dsRNAs bearing sequences of relevant pathogen genes can process them into siRNAs which will block the progress of the infection by specifically attacking the pathogen. The efficiency of the strategy depends on the capacity of siRNAs to silence the target gene or genes that are essential for the pathogen development or infection. For viruses, the attack of the pathogen transcripts (the viral genome for RNA viruses) will occur inside the plant cell, whereas the blockage of fungi or insects will depend on the uptake by the pathogen and stability of the siRNAs. Comprehensive lists of successful transgenic crops exhibiting enhanced resistance to insects (Supplementary Table S1), fungi (Supplementary Table S2), nematodes (Supplementary Table S3), and viruses (Supplementary Table S4) are provided.

Exogenous application of dsRNA as a GMO-free approach for crop protection

Alternative methods of exogenous application of RNAs in plants have been developed over the years. These innovative methods include spraying, infiltration, injection, and mechanical inoculation, among others, and they have been widely used for the delivery of dsRNA or mature sRNAs targeting essential or virulence-related pathogen genes (San Miguel and Scott, 2016; Gogoi *et al.*, 2017; Dalakouras *et al.*, 2018).

Spray-induced gene silencing (SIGS) has emerged as a highly appealing alternative for sustainable agriculture (Mitter *et al.*, 2017; Worrall *et al.*, 2019a). Numerous studies have shown that by applying RNAs exogenously, plants can absorb them into their cells. In the case of dsRNA, the endogenous core components for sRNA biogenesis are capable of processing precursors and generating molecules with silencing activity against endogenous transcripts or alternatively against specific RNAs belonging to a plant pathogen, according to the dsRNA design (Zotti *et al.*, 2018; Dalakouras *et al.*, 2020; Cisneros and Carbonell, 2020).

Just as for conventional pesticides, the potential side effects of the application of RNAi-based pesticides on human health and ecosystems need to be assessed. One of the most important risks to be considered is the 'non-target organisms effect'. In this sense, it remains essential that the selection and design of RNA sequences do not affect off-targets (i.e. the designed sequence do not exert effects on genes other than the specific gene of the target pest) (Chen *et al.*, 2020; Hashiro and Yasueda, 2022). To this end, critical steps should be taken into account for the target gene selection and dsRNA design, including *in silico* analyses of sequence homology of the designed dsRNA and the genomic or transcriptomic sequences of relevant organisms using bioinformatic tools and the latest available omics databases (Qiu *et al.*, 2005; Naito *et al.*, 2005).

Protection strategies based on naked exogenous dsRNAs

The first report for exogenous delivery of dsRNA molecules on plants, inducing RNAi of a plant gene, was designed for *Nicotiana benthamiana* in a Monsanto patent published in 2011. In this study, dsRNAs and 21 nt sRNAs could target the endogenous *PHYTOENE DESATURASE* mRNA. Both dsRNAs and mature sRNAs were sprayed on pre-treated plants with the surfactant Silwet L-77, resulting in widespread *PHYTOENE DESATURASE* down-regulation (Sammons *et al.*, 2011).

Since then, several studies have reported the effects of exogenous application of naked dsRNAs to induce the resistance to viruses, insects, and fungal pathogens in various plant species. Notably, tobacco plants have been widely used to investigate the effects of dsRNA foliar application against common plant viruses, such as pepper mild mottle virus (PMMoV) or tobacco mosaic virus (TMV) (Tenllado and Díaz-Ruíz, 2001; Konakalla *et al.*, 2016; Mitter *et al.*, 2017; Niehl *et al.*, 2018), and more recently against the bean common mosaic virus (BCMV; Worrall *et al.*, 2019b). Also, Carbonell *et al.* (2008) analyzed the effects of inoculation with viroid-specific dsRNAs in tomato, gynura, and chrysanthemum. Kaldis *et al.* (2018) used *in vitro* synthesized dsRNAs to evaluate the resistance against zucchini yellow mosaic virus (ZYMV), an important pathogen of *Cucurbitaceae*, in cucumber, watermelon, and squash. More recently, Tabein *et al.* (2020) analyzed the protection efficacy of exogenous application of naked dsRNAs against one of the 10 most economically important viruses in the world, namely tomato spotted wilt virus (TSWV) both in *N. benthamiana* and in tomato. In a similar study, Rego-Machado *et al.* (2020) reported that topical application of dsRNA in *Chenopodium quinoa*, *Nicotiana glutinosa*, and tomato plants induces protection against tomato mosaic virus (ToMV), one of the main factors severely impacting tomato production and cultivation.

For the control of pathogen insects, several experiments served to determine the effectiveness of dsRNA by direct feeding, spraying, or trunk injection. Li *et al.* (2015) observed an increased insect mortality on the Asian corn borer (*Ostrinia furnacalis*, considered one of the most destructive insect pests of maize), after feeding the larvae with a solution of dsRNA and also after soaking the roots or seeds of rice and maize in a solution containing the dsRNA. San Miguel and Scott (2016) reported lowered biological activity of Colorado potato beetle in potato plants treated with dsRNA or after larvae feeding.

Interestingly, Gogoi *et al.* (2017) found dsRNA in different insect species (aphids, whiteflies, and mites) after inoculating a solution of dsRNAs onto the upper side of tomato leaves. Finally, in a recent study, Dalaisón-Fuentes *et al.* (2022) found that injection or ingestion of dsRNA caused a significant reduction in ovipositions and alterations in oocyte development in adult females of *Dalbulus maidis*, the main vector of important stunting pathogens affecting maize production.

Concerning fungal pathogens, Koch *et al.* (2016) reported inhibition of fungal growth in *Fusarium graminearum* (a globally important pathogen of cereals) and weaker disease symptoms in barley. Wang *et al.* (2016) demonstrated that by using *in vitro* synthesized dsRNA against *DCL1* and *DCL2* genes of *Botrytis cinerea* (one of the most important vegetable and fruit pathogens worldwide), the fungal growth and the symptoms were reduced in tomato and strawberry fruits, grape, lettuce, onion, rose, and also the model species *Arabidopsis thaliana*. In a screening study, McLoughlin *et al.* (2018) analyzed 59 target genes of *Sclerotinia sclerotiorum* (an internationally important pathogen that causes disease in a variety of broadleaf crops). By foliar application of dsRNA onto leaf surfaces in oilseed rape and Arabidopsis, the authors found that out of the 59 dsRNAs tested, 20 showed antifungal activity against *S. sclerotiorum* and *B. cinerea*. Song *et al.* (2018) demonstrated the antifungal activity of the dsRNA designed against the *Myosin 5* gene of *Fusarium asiaticum* (widely reported as the major causal agent of Fusarium head blight of cereals) by spraying the dsRNAs in wheat. Similarly, Gu *et al.* (2019) observed antifungal activity against the same phytopathogen and others (*B. cinerea*, *Magnaporthe oryzae*, and *Colletotrichum truncatum*) by applying a dsRNA to target the *β2Tub* gene in cucumber, soybean, barley, and wheat. More recently, Duanis-Assaf *et al.* (2022) reported that by targeting three essential transcripts active in the fungal ergosterol biosynthesis pathway (dsRNA-ERG) of *B. cinerea*, germination and growth were decreased in *in vitro* conditions and in various fruits and vegetables, including onion, cultivated pink rose, strawberries, red bell-peppers, cherry, mango, and grapes.

Large-scale production of dsRNAs

Although the exogenous application of dsRNA emerges as a promising strategy to develop new tools for crop protection against a plethora of pathogens, the production of dsRNA could be expensive, thus limited to a small scale. The current methods for dsRNA production can employ either *in vitro* or *in vivo* systems.

In vitro systems are based on enzymatic transcription or chemical synthesis (Voloudakis *et al.*, 2015). The enzymatic transcription approach uses PCR-generated templates with specific primers including a specific promoter at the 5′ end of the amplicon that allows the subsequent transcription with a DNA-dependent RNA polymerase from bacteriophage T3, T7, or SP6, respectively, to produce short and long dsRNA molecules. However, as we mentioned before, these commercial kits are quite expensive for the synthesis of large amounts of dsRNA. For example, it could represent up to US$60 per gram of dsRNA (Zotti *et al.*, 2018; Dalakouras *et al.*, 2020). On the other hand, by using chemical synthesis, it is possible to produce a large yield of high purity dsRNA, but the cost is considerably higher for large dsRNA molecules. Interestingly, this approach allows the control of the the quantity and purity, and the addition of chemical modifications of the dsRNA, thus improving the stability and delivery of the molecule (Beaucage and Reese, 2009; Ahmadzada *et al.*, 2018).

In vivo approaches emerge as a good alternative to overcome the problem of the expensive consumables used for dsRNA production. Genetically engineered bacteria such as *Escherichia coli* and *Pseudomonas syringae* and the yeast *Yarrowia lipolytica* are able to produce large amounts of dsRNA molecules at a low cost (Voloudakis *et al.*, 2015; Álvarez-Sánchez *et al.*, 2018). Over the last few years, researchers and companies have devoted efforts to develop new strategies for a suitable microbial-based dsRNA production to meet the growing demand for RNA designed for agriculture (Shew *et al.*, 2017; Zotti *et al.*, 2018; Dalakouras *et al.*, 2020). However, the use of genetically modified microbes may require additional controls for regulatory approvals, in contrast to cell-free RNA synthesis.

Chemical modifications for naked dsRNA stabilization

A promising approach for naked dsRNA stabilization in insect management is based on chemical modifications in the ribose-phosphate backbone of the long dsRNA. It was proposed that these modifications protect the RNA from degradation in insect gut and environmental nucleases. For example, Gong *et al.* (2011, 2013) enhanced the silencing effectivity of siRNAs by adding two 2′-methoxy-nucleotides on each end to control *Plutella xylostella*, the main pest of *Brassicaceae* crops. Recently, Hunter and Wintermantel (2021) incorporated modified pyrimidines 2′F-U and 2′F-C in a dsRNA. The results demonstrated an enhanced RNAi activity, measured as a significant increase in insect mortality (12–35% greater than non-modified dsRNA). Interestingly, the authors also obtained similar results in different insect species such as the Asian citrus psyllid (*Diaphorina citri*, Liviidae) the vector of the bacterium Huanglongbing (HLB) considered the most important disease of citrus worldwide; whitefly (*Bemisia tabaci*, Aleyrodidae), a relevant pest on cotton and vegetables; and the glassy winged sharpshooter (*Homalodisca vitripennis*, Cicadellidae), an important insect vector of the xylem-limited bacterial plant pathogen *Xylella fastidiosa*. Also, Howard *et al.* (2022) reported that dsRNAs containing phosphorothioate modifications exhibit increased resistance to Southern green stink bug (*Nezara viridula*, an economically important crop insect pest) saliva

nucleases, increased efficacy in *Drosophila melanogaster* cell cultures, and increased mortality in both stink bug (*Halyomorpha halys*, which causes major economic damage to fruit, vegetable, and field crops in the mid-Atlantic region) and corn rootworm (*Diabrotica virgifera virgifera*, one of the most economically important pests of corn in the USA). These results demonstrate the high potential of chemical modifications of dsRNAs for insect pest control in crops.

Although all these studies reported effectiveness against multiple pathogens in several plant species, fruits, and vegetables, the protection period was variable and dependent on multiple factors. In particular, the instability of the naked dsRNAs applied is likely to result in short periods of plant protection. Also, the limited mobility of naked dsRNA could represent several issues in the large-scale use of naked dsRNA for crop and postharvest protection.

Nanotechnology for the stabilization of exogenous dsRNA

By applying free dsRNA in an aqueous solution, the transcripts exhibit a very low protection permanence (of up to a few days) due to the instability of these molecules, being a substrate for a great variety of non-specific RNases from the plant, as well as from different microorganisms. This method of application faces similar issues to pesticides, which exhibit short longevity due to environmental degradation, and difficulties with site-specific uptake by the targeted pest (Dalakouras et al., 2016, 2018; Mitter *et al.*, 2017; Dubrovina and Kiselev, 2019).

The effectiveness of RNAi falls significantly depending on the delivery approach. Therefore, several studies have been focused on the development of alternative nanoparticles (NPs) to improve the resistance and stability of dsRNAs against abiotic factors and the enzymatic degradation in the field (Jiang *et al.*, 2014; Numata *et al.*, 2014; Avila *et al.*, 2018; Serrano-Sevilla *et al.*, 2019). A comprehensive list of successful RNA-based technologies and the related engineered encapsulation systems suitable for crop protection, improving the transport and protection of dsRNA, can be found in Table 1.

NPs include particles ranging in size from 1 nm to 100 nm (Kumar *et al.*, 2018). Synthetic, non-toxic NPs can be generated from natural as well as synthetic materials including metals, cationic polymers, and lipids, among others, with wide spectrum functions and applications (Blanco *et al.*, 2015). Using NPs for RNAi-based crop protection offers advantages such as a high degree of RNA encapsulation, biodegradability, and promoted penetration (Herrero-Vanrell *et al.*, 2005; Bamburowicz-Klimkowska *et al.*, 2019). Also, NPs may protect dsRNA molecules against UV radiation and free or plant cell non-specific nucleases that can degrade the naked dsRNA molecules (Christiaens *et al.*, 2020). An increasing range of NPs have been developed to protect dsRNA molecules against degradation without affecting their ability to silence genes (Dubrovina and Kiselev, 2019; Dalakouras *et al.*, 2020). In addition to the stabilization of the RNAs on the plant surface, NPs should promote a gradual and sustained release of the active biomolecules over time. In particular, the efficiency of siRNAs against pathogens has been enhanced through formulations of dsRNA precursors with NPs of different natures, including liposome, chitosan, clay, and guanylate (Vélez and Fishilevich, 2018; Christiaens *et al.*, 2020). In recent years, important advances have been made in this field, including the use of different NPs that stabilize RNA molecules and allow a gradual and sustained release over time (Fig. 2).

Nanoparticles of chitosan and LDH (layered double hydroxide)

Most of the experimental assays involving NPs have been designed to study the effects of exogenous RNAs on insect pests. One of the first studies involving NPs and dsRNA was reported by Zhang *et al.* (2010). It was demonstrated that feeding mosquito larvae with the dsRNAs *AgCHS1* and *AgCHS2* loaded onto chitosan NPs increased the larval susceptibility to diflubenzuron, and calcofluor white (CF) or DTT. He *et al.* (2013) fed the lepidopteran pest, Asian corn borer (*Ostrinia furnacalis*), with a diet containing a mixture of a fluorescent nanoparticle (FNP) and CHT10-dsRNA; naked CHT10-dsRNA; FNP and green fluorescent protein (GFP)-dsRNA; and GFP-dsRNA. The results served to show that the NP was efficient in entering into live cells with low cytotoxicity and high gene delivery efficacy. This study was the first in which a non-viral gene delivery system was used as a tool for the genetic control of insect pests. Over the last few years, several studies involving chitosan NPs and dsRNA have been conducted mostly in the malaria mosquito, *Anopheles gambiae* (David *et al.*, 2013; Mysore *et al.*, 2014; Zhang *et al.*, 2015; Dhandapani *et al.*, 2019). This line of research allowed the development and refinement of these nanocomplexes and showed that by using chitosan NPs, RNAi efficiency in insects was improved. Based on these previous works, Gurusamy *et al.* (2020) demonstrated that conjugating dsRNA with chitosan NPs helps the dsRNA to escape from endosomes in the fall armyworm, *Spodoptera frugiperda*, an important harmful pest for corn crops. In a similar study, Kolge *et al.* (2021) reported that chitosan NPs+dsRNA complexes were stable on the surface of chickpea leaves at least 5 d after inoculation, and their ingestion effectively silenced *JHAMT* and *ACHE* genes and caused 100% insect mortality in *Helicoverpa armigera*, a devastating pest of cotton and other important crops. Collectively, these findings suggest that chitosan formulation to protect dsRNA for field application is a suitable technology with high potential for pest and disease control. Chitosan interacts electrostatically with dsRNA (Zhang *et al.*, 2010). The resulting structure can aid the endosomal escape of dsRNA and can consequently increase the RNAi efficiency for crop protection. Also, dsRNA+chitosan encapsulation efficiency results

Table 1. Successful applications of non-stabilized and stabilized dsRNA against several organisms

Delivery method	Target	Application method	Effect	Plant host	Reference
Naked	*RP* gene of PMMoV	Mechanical inoculation or spraying with atomizer	Resistance to PMMoV	Tobacco	Tenllado and Díaz-Rúiz (2001)
Naked	*p126* and *CP* genes of TMV	Mechanical inoculation	Resistance to TMV	Tobacco	Konakalla *et al.* (2016)
Naked or loaded into LDH	*RP* gene of PMMoV; *2b suppressor* gene of CMV2b	Spraying	Resistance to PMMoV and CMV	Tobacco, cowpea	Mitter *et al.* (2017)
Naked	*RP* gene of TMV; *GFP* gene of TMV	Mechanical inoculation; spraying	Resistance to TMV	Tobacco	Niehl *et al.* (2018)
Naked	*Nib* and *CP* genes of BCMV	Spraying	Resistance to BCMV	Tobacco, cowpea	Worrall *et al.* (2019b)
Naked	Viroid-specific dsRNAs	Mechanical inoculation	Resistance to PSTVd, CEVd, and CChMVd	Tomato, gynura, and chrysanthemum	Carbonell *et al.* (2008)
Naked	*HC-Pro* and *CP* genes of ZYMV	Mechanical inoculation	Resistance to ZYMV	Cucumber, watermelon, and squash	Kaldis *et al.* (2018)
Naked	Nucleocapsid (N) or the movement protein (NSm) of TSWV	Mechanical inoculation; spraying	Resistance to TSWV	Tobacco, tomato	Tabein *et al.* (2020)
Naked	*CP* gene of ToMV	Spraying	Resistance to ToMV	Tobacco, quinoa, tomato	Rego-Machado *et al.* (2020)
Naked	*Cyp18A1* and *Ces* genes of BPH; *KTI* gene of ACB	Root or seed soaking; larvae feeding	Increased insect mortality rate	Rice, maize	Li *et al.* (2015)
Naked	*Actin* gene of CPB	RNA dropped on leaf surface; larvae feeding	Lowered biological activity of CPB	Potato	San Miguel and Scott (2016)
Naked	*HC-Pro* gene of ZYMV	Mechanical inoculation	dsRNA detection in tomato and in insects	Tomato	Gogoi *et al.* (2017)
Naked	*Bicaudal C (BicC)* gene of *D. maidis*	Injection, adult feeding	Significant reduction in transcription and ovipositions	No	Dalaisón-Fuentes *et al.* (2022)
Naked	*CYP51A*, *CYP51B*, and *CYP51C* genes of *Fusarium graminearum*	Spraying	Inhibition of fungal growth and weaker disease symptoms; suppression of target fungal *CYP51* mRNAs	Barley	Koch *et al.* (2016)
Naked	*DCL1* and *DCL2* genes of *Botrytis cinerea*	RNA dropped on leaf surface	Inhibition of fungal growth and weaker disease symptoms; suppression of fungal *DCL* transcripts	Tomato, strawberry, grape, lettuce, onion, rose, Arabidopsis	Wang *et al.* (2016)
Naked	59 target genes of *Sclerotinia sclerotiorum*	Spraying	20/59 genes showed antifungal activity and weaker disease symptoms; suppression of fungal target genes	Oilseed rape, Arabidopsis	McLoughlin *et al.* (2018)
Naked	*Myosin 5* gene of *Fusarium asiaticum*	Spraying	Weaker disease symptoms; suppression of fungal *Myo5* transcript levels	Wheat	Song *et al.* (2018)
Naked	β*2Tub* gene of *Fusarium asiaticum*	Mechanical inoculation	Antifungal activity against *F. asiaticum*, *B. cinerea*, *Magnaporthe oryzae*, and *Colletotrichum truncatum* and weaker disease symptoms	Cucumber, soya, barley, wheat	Gu *et al.* (2019)
Naked	*ERG*13, *ERG11*, and *ERG*1 of *B. cinerea*	Spraying	Antifungal activity against *B. cinerea* and weaker disease symptoms	Onion skin, rose petals, strawberry, Bell-pepper, cherry, mango, grape, tomato	Duanis-Assaf *et al.* (2022)

Table 1. Continued

Delivery method	Target	Application method	Effect	Plant host	Reference
Chemical modification	*siRNA* against *Rieske* iron–sulfur protein (*RISP*) gene of *Plutella xylostella*	Larvae feeding	Lower transcript levels of *RISP* compared with the control	NO	Gong *et al.* (2011)
Chemical modification	*AChE1* and *AChE2* genes of *Plutella xylostella*	Spraying	Reduced transcript levels of *AChE2*	*Brassica oleracea* and *Brassica alboglabra*	Gong *et al.* (2013)
Chemical modification	*Syntaxin-1A* gene of *Asian citrus* psyllid (*Diaphorina citri*); whitefly (*Bemisia tabaci*); and the glassy-winged sharpshooter (*Homalodisca vitripennis*)	Spraying	Increased insect mortality by 12–35%	Lemon plant	Hunter and Wintermantel (2021)
Chemical modification	Genes of *Drosophila melanogaster*, *Nezara viridula*, *Halyomorpha halys*, *Diabrotica virgifera*	Feeding	Increased resistance to southern green stink bug saliva nucleases	NO	Howard *et al.* (2022)
Chitosan nanoparticles	*AgCHS1* and *AgCHS2* of *Anopheles gambiae*	Feeding	Increased the larval susceptibilities to pesticides	No	Zhang *et al.* (2010)
Chitosan nanoparticles	Fluorescent nanoparticle (FNP)	Feeding	NP was efficient to enter into live cells with low cytotoxicity and high gene delivery efficacy	No	He *et al.* (2013)
Chitosan nanoparticles	Luciferase, inhibitor of apoptosis (*iap*) of *Spodoptera frugiperda*	Feeding	Increased levels of mortality (47%)	No	Gurusamy *et al.* (2020)
Chitosan nanoparticles	*JHAMT* and *ACHE* genes of *H. armigera*	Feeding and spraying	100% insect mortality	Chickpea	Kolge *et al.* (2021)
LDH nanoparticles	*CMV2b* and *VIR54* genes of CMV or PMMoV	Spraying	Virus protection	Cowpea, tobaco	Mitter *et al.* (2017)
LDH nanoparticles	*Nib* and *CP* genes of BCMV	Spraying	Resistance to BCMV	Tobacco, cowpea	Worrall *et al.* (2019b)
LDH nanoparticles	*ERG13*, *ERG11* and *ERG1* of *B. cinerea*	Spraying	Antifungal activity against *B. cinerea* and weaker disease symptoms	Onion skin, rose petals, strawberry, bell-pepper, cherry, mango, grape, tomato	Duanis-Assaf *et al.* (2022)
LDH nanoparticles	*Ace1*, *AQP1*, *Vhaa* and *zfp* genes of whitefly	Feeding; spraying	75–96% mortality	Cotton, tomato	Jain *et al.* (2022)
LDH nanoparticles	*DCL1* and *DCL2* genes of *Botrytis cinerea*	Spraying	Antifungal activity against *B. cinerea* and weaker disease symptoms	Tomato	Niño-Sanchez et al. (2022)
Guanidine-containing polymers	*V-ATPase* gene of *Spodoptera frugiperda*	Feeding	Increased mortality (53%)	No	Christiaens *et al.* (2018)
Guanidine-containing polymers	*Chitin synthase B* gene of *Spodoptera frugiperda*	Feeding	80% transcript reduction and 30% reduction of larval and pupal mortality	No	Parsons *et al.* (2018)
Peptide or protein-based nanoparticles	*CHS2* gene of *Anthonomus grandis*	Feeding	80% of transcript reduction	No	Gillet *et al.* (2017)
Peptide or protein-based nanoparticles	*BiP* and *Armet* gene of *Tribolium castaneum*; *BiP* of *Acyrthosiphon pisum*	Feeding	Significant increase in mortality	No	Avila *et al.* (2018)

Table 1. Continued

Delivery method	Target	Application method	Effect	Plant host	Reference
Lipid vesicles	*RPS13* and *VHA26* gene of *Drosophila suzukii*	Feeding	Significant increase in mortality	No	Taning *et al.* (2016)
Lipid vesicles	α-*tubulin* gene of *Blattella germanica*	Feeding	Significant increase in mortality	No	Lin *et al.* (2017)
Lipid vesicles	*tub* gene of *Blattella germanica*	Feeding	Significant increase in mortality	No	Huang *et al.* (2018)
Lipid vesicles	*vATPase A* and *actin* gene of *Euschistus heros*	Feeding	45% increased mortality	No	Castellanos *et al.* (2019)
Bacterial vesicles	*DIAP1* gene of *Henosepilachna vigintioctopunctata*	Feeding	Reduced transcript levels	No	Hashiro *et al.* (2019)
Bacterial vesicles	*CP* gene of PVX	Spraying	Higher level of virus protection (60%)	Tobaco	Necira *et al.* (2021)
Bacterial vesicles	*Chs3a*, *Chs3b*, and *DCL1* and *DCL2* genes of *Botryotinia fuckeliana*	Spraying	Antifungal activity against *Botryotinia fuckeliana* and weaker disease symptoms	Strawberry	Islam *et al.* (2021)
Bacterial vesicles	*GFP* gene	Injection, adult feeding	Reduced GFP signal	No	Whitten *et al.* (2016)

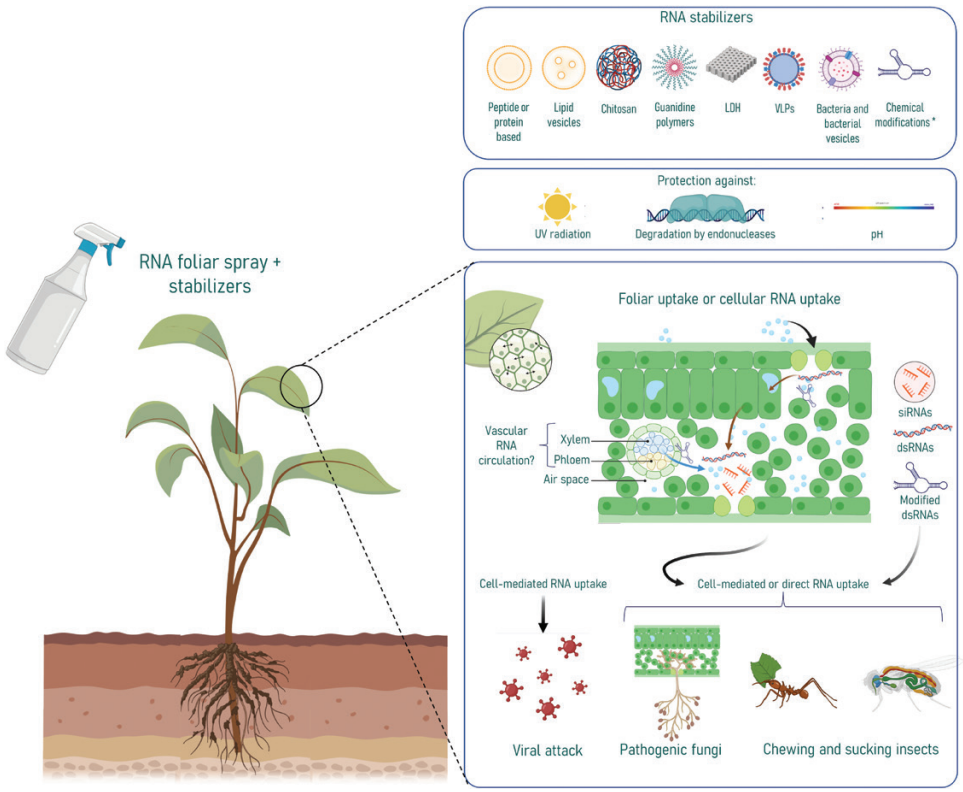

Fig. 2. Exogenous RNA-mediated crop protection by SIGS. Nanoparticle-mediated dsRNA or siRNA delivery systems can protect RNAs against several factors affecting the stability of RNA, such as degradation by UV light, endonucleases from different organisms, pH, and others. Sprayed RNAs (dsRNAs or mature sRNAs) can be distributed in plant tissues via cellular uptake. In foliar uptake, the sprayed dsRNAs on the leaf surface enter the leaf epidermal cells. Once here, the dsRNAs molecules can move through vascular bundles to other parts of the plant and then they can be directly taken up by different target pathogens (insects and pathogenic fungi) and trigger the RNAi response. Also, fungi and insects may incorporate the dsRNAs directly from the plant surface. Alternatively, cellular uptake may imply the penetration of the dsRNAs into the plant cell cytoplasm where the RNAi machinery can process dsRNAs into siRNAs. Then, the produced siRNAs can trigger the degradation of viral transcripts and protect the plant against the viral attack or be taken up by other target organisms (insects and pathogenic fungi) and trigger the RNAi response. *Chemical modifications can also stabilize RNA and protect it from pH variations and degradation, especially in insects. Modified from Hoang *et al.* (2022). Figure was created with BioRender.com.

improved when sodium tripolyphosphate is used as a cross-linker (Dhandapani *et al.*, 2019). Further research focused on chitosan+dsRNA formulations may allow the development of RNAi-based technology for crop protection.

In addition to chitosan, LDH (layered double hydroxide) NPs have received plenty of interest for dsRNA nanoencapsulation due to their biocompatibility, low toxicity, and biodegradability. LDHs, also referred to as hydrotalcite-like compounds (HTlcs), constitute a large group of natural and synthetic minerals whose physico-chemical properties have strong analogies with clay with those of cationic clay minerals (Forano *et al.*, 2013). LDH forms a positively charged structure with the dsRNA, then the LDH material is slowly degraded by environmental conditions and provides sustained release of dsRNA on the leaf, and protects the dsRNA from leaf surface run-off and from metabolic breakdown (Mitter *et al.*, 2017). Spraying of dsRNAs loaded onto LDH clay nanosheets led to a successful antiviral effect in the plant for at least 20 d, and the dsRNA remained detectable on treated leaves up to 30 d after application (Mitter *et al.*, 2017). Worrall *et al.* (2019b) used LDH NPs to load a dsRNA against BCMV. According to their results, plants treated with the LDH NPs+dsRNA were not infected by the virus, while non-treated plants tested positive for BCMV. In a recent work, Duanis-Assaf *et al.* (2022) reported that LDH+dsRNA complexes can inhibit the fungal growth of *B. cinerea*. Interestingly, the potency of the applied solution was effective for at least 6 weeks in cold storage and significantly reduced the development of gray mold in grapes. A similar result was reported by Niño-Sánchez et al. (2022), who found that LDH+dsRNA provided prolonged protection in tomato plants. Also, Jain *et al.* (2022) demonstrated that the application of dsRNA loaded onto LDH effectively disrupts multiple whitefly developmental stages *in planta*. These results highlighted the protection effect of this technology against viruses transmitted by vector insects.

Guanidine-containing polymers

Another less explored nanocarrier for dsRNA in crop protection consists of guanidine-containing polymers which have been designed for the protection of dsRNA over variations in the pH found in the gut of insect pests. These polymers allow the escape of RNA from endosomes and the endocytic passage through cell membranes (Chen *et al.*, 2012). Christiaens *et al.* (2018) demonstrated that dsRNA loaded on guanidine-containing polymer NPs led to an increased mortality (53%) in the beet armyworm *Spodoptera exigua* (one of the most important worldwide pest of vegetables) and completely halted the development of the caterpillars. In a similar study, Parsons *et al.* (2018) showed that feeding second and third instar larvae of the fall armyworm *S. frugiperda* with a guanidine-containing polymer–dsRNA complex resulted in an 80% transcript reduction and a 30% reduction of larval and pupal mortality even at 29 d post-treatment. Altogether, these studies demonstrated the protective effect that guanidine functional groups provided to dsRNA and the potential of this compound to be used in insect pest control using dsRNA-based technology.

Peptide- or protein-based nanoparticles

Peptide- or protein-based NPs have also been used as dsRNA delivery vehicles, especially against insects. Gillet *et al.* (2017) showed that combining a chimeric protein PTD–DRBD (peptide transduction domain–dsRNA-binding domain) with dsRNA allows the formation of a ribonucleoprotein (RNP) particle that enhances the effectiveness of the RNAi mechanism in the cotton boll weevil *Anthonomus grandis*. Branched amphiphilic peptide capsules (BAPCs) have a similar structure to liposomes, but with greater stability (Wessel *et al.*, 2019). The first application of this peptide-based technology for dsRNA delivery was demonstrated by Avila *et al.* (2018) in the pea aphid *Acyrthosiphon pisum* (a major pest of pea, lucerne, and clover) and the red flour beetle *Tribolium castaneum* (a major pest causing significant losses of agrifood commodities during post-harvest storage). In their report, they found that BAPC/BiP–dsRNA complexes induced mortality in both species with high effectivity compared with naked dsRNA. The results of these investigations are promising for the development and testing of peptides and proteins for their application in dsRNA-based crop protection.

Lipid vesicles

Another potential strategy for RNA delivery to plant pests and pathogens is the use of lipid vesicles. Liposome-encapsulated dsRNA has been used to introduce siRNA precursors to insect species lacking systemic RNAi responses, such as *Drosophila suzukii* (an important pest that causes major damage in fruit production). Taning *et al.* (2016) demonstrated that by encapsulating *rps13* and *vha26* dsRNA in a liposome, *D. suzukii* larvae and adults exhibited a significant increase in mortality, whereas naked dsRNA did not show any effect. Lin *et al.* (2017) reported that oral delivery of the dsRNAs encapsulated by liposomes in the German cockroach *Blattella germanica* caused dramatic depletion of the essential α-tubulin gene, thus increasing mortality. Also, Huang *et al.* (2018) demonstrated that the *tub* dsRNA encapsulated with liposome carriers was able to induce death in the cockroach *B. germanica* with high effectiveness. More recently, Castellanos *et al.* (2019) reported that liposome-encapsulated dsRNA targeting vATPase A and muscle actin increased mortality by 45% in the neotropical stink bug *Euschistus heros*, an important pest in many crops.

Imitating the naturally occurring RNA exchange pathways in plants could be a promising tool for improving the strategy of delivery of RNA against plant pests and pathogens.

For example, Cai *et al.* (2019) discovered that plants use extracellular vesicles to deliver sRNAs into interacting fungal pathogens to silence fungal virulence-related genes. The results of the study served to demonstrate that extracellular vesicles (EVs) secreted from Arabidopsis cells containing sRNA are efficiently taken up by *B. cinerea* fungal cells, thus silencing fungal genes critical for pathogenicity. More recently, He *et al.* (2021) found that a set of RNA-binding proteins, including AGO1, RNA helicases, and annexins, were present in EVs of Arabidopsis. These results are highly promising for the development of new technologies focused on fungal pathogens controlled by dsRNA-based delivery even in those lacking their own RNAi machinery.

Virus-like particles (VLPs)

Virus-like particles (VLPs) are molecular vehicles derived from key structural components of viral origin that have been repurposed to deliver a cargo different from the initial viral genome (Ludwig and Wagner, 2007; Zepeda-Cervantes *et al.*, 2020). One of their most relevant properties is the capacity for packaging of foreign RNA. A VLP-based technology has been developed and probed against ants in a patent by Killmer *et al.* (2017). The advantages of using VLPs in agricultural biotechnology remain in the efficient cellular uptake and high degree of protection of the dsRNA in extracellular environments. However, further research is needed to explore the effectiveness of this technology against different pathogens and pests.

Bacterial vesicles

dsRNA can also be encapsulated in bacteria. This approach has been demonstrated to be effective against insects, suggesting that bacterial cell packaging may act as a stabilizer of dsRNA in the lumen of the digestive system of insects. For example, Hashiro *et al.* (2019) fed the ladybird beetle (*Henosepilachna vigintioctopunctata*, an agricultural pest of potato, tomato, and eggplant) with an engineered *Corynebacterium glutamicum* (overproducer of a dsRNA target of the gene *DEATH-ASSOCIATED INHIBITOR OF APOPTOSIS 1*, *DIAP1*). The authors found that expression of the gene target in the pest was suppressed, and the leaf-feeding activity of the larvae was also decreased. Necira *et al.* (2021) reported that application of *E. coli*-encapsulated dsRNA is effective for protection of *N. benthamiana* against the potato virus (one of the most important aphid-transmitted viral pathogens of potato worldwide). Also, Islam *et al.* (2021) reported that *E. coli*-derived minicells could be used for dsRNA production and encapsulation. This research showed that the inoculation of the strawberry surface with minicells carrying a dsRNA against *Botryotinia fuckeliana* (anamorph: *B. cinerea*) significatively reduced the fungal growth, and also in *in vitro* conditions.

Interestingly, another similar approach involving engineered symbionts of the target pest was shown to be effective for dsRNAs delivery in the western flower thrips *Frankliniella occidentalis* (a polyphagous insect that causes large losses on a wide range of crops and is also a vector for plant tospoviruses) and the kissing bug *Rhodnius prolixus* (a prominent vector of Chagas disease). Whitten *et al.* (2016) observed a systemic RNAi response in both pests fed with an engineered symbiont expressing dsRNA and reported that the observed knockdown phenotypes were also horizontally transmissible.

Conclusions and perspectives

Since its discovery >20 years ago, RNAi has been widely used in crops as a protection tool. Traditionally, RNAi approaches have involved the use of transgenic plants expressing precursor dsRNA against selected targets. The host-induced gene silencing (HIGS) RNAi approach, corresponding to *in planta* endogenous expression of siRNA targeting key genes of the pathogen, is crucial to determining the success of the RNAi technology in crop protection and is the most commonly used (Gebremichael *et al.*, 2021). However, the use of transgenics and GMOs in agriculture has raised considerable scientific and public concerns. In particular, the development of novel elite varieties for many crops has dramatically narrowed down the range of cultivated landraces, exacerbating the impact of dynamic pathogen populations across different environments. Furthermore, the development of novel varieties from traditional breeding as well as from transgenic approaches means that once a new pathogen overcomes the technological barriers, there is then a new need for developing improved seeds. In this sense, exogenous delivery of dsRNA can be considered as an excellent alternative approach. Exogenous RNAs can be applied to local landraces and can be redesigned every time a new pathogenic strain overcomes the previous solution. The large number of successful transgenic events against pathogens (Supplementary Tables S1–S4) can serve as a source of valuable information for the design of exogenous RNA tools. Moreover, the growing knowledge of long non-coding RNAs involved in epigenetics (Lucero *et al.*, 2021), alternative splicing (Romero-Barrios *et al.*, 2018), and miRNA kidnapping, among other post-transcriptional regulatory mechanisms of gene expression (Fonouni-Farde *et al.*, 2021), suggests that non-coding transcripts may also be used as biotechnological tools to modulate hormone homeostasis, plant development, and the response to environmental cues. An increasing number of reports suggest that the exogenous delivery method for the application of RNA in the field emerges as one of the most promising approaches to ensure the reduction of chemical compounds used in agriculture and boost productivity in a climate change context (Yin *et al.*, 2009; Dalakouras *et al.*, 2016), constituting a breakthrough for the second green revolution.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. Host-induced gene silencing against insects.

Table S2. Host-induced gene silencing against fungi.

Table S3. Host-induced gene silencing against nematodes.

Table S4. Host-induced gene silencing against viruses.

Author contributions

JR and FA: conceptualization and funding acquisition; JR, FA, and FM: visualization, writing—original draft and writing–review and editing. All authors contributed to manuscript revision, and read and approved the submitted version.

Conflict of interest

The authors declare that they have no conflict of interest.

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