CHAPTER

8 Development of RNAi-based biopesticides, regulatory constraints, and commercial prospects

Aline Koch

Institute of Plant Sciences, Cell Biology and Plant Biochemistry, Plant RNA Transport, University of Regensburg, Regensburg, Germany

Introduction

People worldwide must produce more food and feed more sustainably and eco-consciously than ever. Plant production currently faces complex challenges in light of the climate crisis, biodiversity loss, and rising population growth. In addition to conventional crop protection measures, such as the use of synthetic chemical pesticides, alternative control strategies are necessary, primarily because of environmental protection and the ever-increasing prevalence of resistance to approved active agents among many harmful insects (Alyokhin et al., 2008; Van Leeuwen et al., 2010; Kwon et al., 2016). Restricting available pesticides (e.g., by prohibiting neonicotinoids) exacerbates the problem because active ingredients can only be rotated to a limited extent. Demands for pesticide-free agriculture to protect nontarget organisms and the environment are increasing, particularly in the context of drastic biodiversity loss. Heavy reliance on synthetic chemical pesticides has detrimentally affected the environment and is considered one of many primary causes of insect populations' decline (Wagner et al., 2021). To address these challenges, societies are continuously seeking out innovative technology-based solutions. The protection of biodiversity and the prevention of further biodiversity loss are issues with broad social and political relevance anchored in the European Green Deal (EC, 2019) as well as bioeconomic strategies implemented across the European Union (EU) (EC, 2018; EC, 2020) and United Nations (FAO, 2018; CBD/WG, 2020). The EU's demand that its member countries reduce conventional chemical pesticides by 50% by 2030 will further limit the availability of pesticide substances. Therefore, robust, innovative solutions are urgently needed.

Double-stranded RNA-based biopesticides provide a new and unique mode of action by utilizing naturally occurring RNA interference (RNAi; Fire et al., 1998) to downregulate (silence) essential gene products in target pathogens and pests, causing mortality.

Development and Commercialization of Biopesticides. https://doi.org/10.1016/B978-0-323-95290-3.00013-3

[Info-Box. RNAi denotes gene regulation mechanisms in which short, non-coding RNA molecules suppress the expression of complementary target genes (gene silencing). Today, RNAi-based plant protection technologies exploit post-transcriptional gene silencing molecular mechanisms. RNAi-mediated post-transcriptional gene silencing begins with the breakdown of a double-stranded RNA (dsRNA) precursor into 21−24 nucleotide (nt)-long small interfering (si)RNA duplexes by the RNase−III-like enzyme Dicer. Posttranscriptional gene silencing takes place in the cytoplasm of the cell, where the double-stranded siRNAs are incorporated into an RNA-induced silencing complex (RISC). This multienzyme complex contains an Argonaute protein, which has both an RNA-binding domain and endonucleolytic activity. In an ATP-dependent reaction, siRNA is removed by a RISC, which results in one sense and one antisense strand. While the sense strand, which has the same sequence as the cell's own mRNA, is degraded, the antisense strand remains bound to the RISC and targets complementary mRNA transcripts for degradation.**]**

Sequence-based active ingredients, such as dsRNAs, represent a powerful (and arguably improved) alternative to conventional chemical pesticides by their high target specificity and the resulting protection of nontarget organisms: "Using RNAi as opposed to conventional pesticides is the difference between using tweezers to cripple part of one particular insect versus taking a hammer to a whole row of bugs" (Shaffer, 2020).

Numerous studies have already demonstrated the considerable effectiveness of RNAi-based crop protection technologies for controlling pathogens and harmful insects in agriculture and horticulture (for review, see: Koch and Kogel, 2014; Rosa et al., 2018; Zotti et al., 2018; Gaffar and Koch, 2019; Dalakouras et al., 2020; Liu et al., 2020; Rank and Koch, 2021). Using genetically modified (GM) plants expressing dsRNAs in a process known as host-induced gene silencing (Nowara et al., 2010) (Fig. 8.1), pests can be controlled with average effectiveness (disease resistance/mortality rate) of 90% for viruses, 50% for insects, 59% for fungi, and 56% for nematodes (Koch and Wassenegger, 2021). These promising figures have pushed the development of GM RNAi plants to market maturity (De Schutter et al., 2022). The first GM RNAi product, the corn SmartStax PRO, was approved by US authorities in 2017 and by Chinese authorities in 2021. This GM corn expresses various proteins combining different modes of action to confer tolerance to herbicides (glyphosate and glufosinate-ammonium) and coleopteran and lepidopteran pests (Head et al., 2017). The targeting of corn rootworms (*Diabrotica* spp.) is mediated by the expression of 240 bp dsRNA (MON 87411) designed to match the sequence of the western corn rootworm *Snf7* gene (DvSnf7). The combination (stack) of transgenic insecticidal proteins and RNAi to fight corn rootworms may drastically reduce the number of resistant survivors in a targeted pest population (Shaffer, 2020). SmartStax PRO will be available to farmers in the US from 2022. In Europe, SmartStax PRO has marketing approval for all uses (e.g., imports of products containing MON 87411), excluding cultivation (EFSA, 2019).

While the cultivation and consumption of GM crops are established in large parts of the world (with the United States, Brazil, Argentina, Canada, and India accounting for 91% of global GMO cultivation), in Europe, except for Spain and Portugal, GM crops are not cultivated (ISAAA, 2019). In addition to the negative public perception of GM organisms, the generation of GM plants is laborious, complicated, and limited by a crop's transformability; furthermore, GM approval times and costs are deterrents to small and medium-sized enterprises. For these reasons, exogenous, GM-free application of dsRNA—a more affordable technology—was explored. Subsequently, the GM-free technique known as spray-induced gene silencing, which triggers RNAi by spraying dsRNAs onto plants, was established (Dalakouras et al., 2016; Koch et al., 2016). Both pathogens and pests can take up these dsRNAs from both plant surfaces and tissues with very promising results (Koch and Petschenka, 2022). However, since the first RNAi GM plant entered the market, market testing began for the first exogenous dsRNA-based pesticide (De Schutter et al., 2022).

Specific regulations for RNAi-based products: the risks and requirements?

RNAi-based products share a common, well-studied mode of action: they interfere with gene expression mainly posttranscriptionally at the level of mRNA translation (Fig. 8.1). The exploitation of the RNAi mechanism allows protein production to be blocked via targeted mRNA degradation, thereby preventing target proteins from fulfilling their biological function. In addition to the wide use of dsRNA active ingredients in pathogen and pest control, they may be exploited to target endogenous genes to modify plant metabolism, growth, and development. Comprehensive and

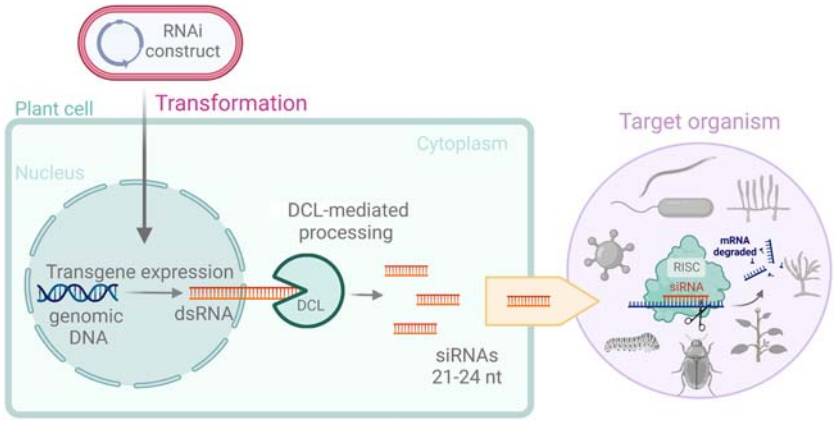

FIGURE 8.1

Mechanism of host-induced gene silencing.

Created with BioRender.com.

extensive studies have targeted endogenous genes with exogenous siRNA application (Dalakouras et al., 2016; Schwartz et al., 2020; Zhang et al., 2020, 2021; Dalakouras and Ganopoulos, 2021; Hendrix et al., 2021). These studies have laid a foundation for the endogenous modification of crop plants beyond targeting their interacting pests. For example, abiotic stress resistance, nutritional value, harvest control, and aesthetic properties (e.g., color, taste, and shape), could be adapted to improve crop traits, performance, and yield. Moreover, RNAi is widely used for unraveling gene function through reverse genetics; thus, exogenous RNA applications may also be useful for basic research. Based on these examples, RNAi's potential applications seem versatile. Still, they may be limited as understanding the prerequisites for achieving a full-fledged RNAi response following external dsRNA application improves.

Recent advances in commercialization—for instance, the approval of the first RNAi GM plant in 2018—have increased the need for guidance on the regulation of RNAi-based pesticides. To this end, several organizations and agencies have developed a broad set of recommendations examining their potential risks (USEPA, 2013; Christiaens et al., 2018, EFSA supporting publication; Dávalos et al., 2019, EFSA supporting publication; OECD, 2020). The most recent considerations and recommendations on regulating exogenously applied dsRNA-based products derive from the Organization for Economic Co-operation and Development (OECD) working paper "Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides" (OECD, 2020), some major aspects of which are highlighted in the following sections.

Sources of potential off-target effects

Given that RNA-based pesticides will likely be applied using the same methods as conventional chemical pesticides for outdoor (e.g., field) and indoor (e.g., greenhouse) use, they may fall under the same (country-specific) regulatory framework. However, particular attention is required to emphasize the major differences between RNA-based pesticides and conventional chemical pesticides and the former's unique characteristics to enable regulatory agencies to adapt to their risk assessments. The sequence-based mode of action of dsRNA active ingredients is a unique feature with both positive and negative repercussions. On the one hand, the sequence of a chosen dsRNA is specific to its pathogenic mRNA cognate; on the other hand, it represents a source of potential risk based on its effect on siRNAs sufficiently short of causing off-target effects, such as unintentional gene silencing. These potential off-target effects present risks to nontarget organisms and must be evaluated by registrants or applicants and risk assessors. In general, off-target gene silencing can occur in both target and nontarget organisms; however, only adverse effects on nontarget organisms are unintentional because both on- and off-target gene silencing cause reduced fitness or mortality in the target organisms to produce the desired effect. It is important to note that off-target gene silencing in nontarget organisms will not inevitably affect mortality, reproduction, or growth; it may cause effects of no

consequence that are not discernible or testable, and it is, therefore, difficult to predict whether effects will be adverse or negligible.

The fundamental basis for both avoidance and prediction of off-target effects are in silico comparisons used to identify sequence similarities between RNA sequences present in a target and nontarget organism and all siRNAs that may derive from a dsRNA-based pesticide. Notably, the OECD working paper stressed that "critical to the application of sequence analysis in assessing the likelihood of off-target effects of dsRNA products is an understanding of the degree of sequence similarity necessary for effective RNA-mediated gene suppression, as well as an understanding of several structural and biological factors which can affect the binding of small RNAs to mRNA" (OECD, 2020). It is essential to clarify that, although sequence alignment represents one major source of off-target effects, it does not invariably confer risk to off-target organisms. Sequence alignment merely indicates that one prerequisite for being affected by RNAi (sequence complementarity) has been fulfilled. Off-target predictions based on in silico analysis cannot be regarded as adequate, standalone risk assessments (OECD, 2020). Because there is a lack of reliable data on the accuracy of in silico off-target prediction, which can be assessed via in vivo off-target effects and the percentage of adverse events, more effort should be invested in generating sufficient data and further developing in silico predictions. For example, prediction tools that consider differences in the degree of complementary and other specific factors that influence silencing efficiency in different organisms are needed. Existing tools should thus be continuously revised to integrate emerging knowledge on species-specific functionalities of diverse RNAi machinery. Moreover, ongoing research is required to understand better the level of sequence similarity and the minimum threshold needed to provoke siRNA-mediated gene silencing among target and nontarget species. Another important factor related to using prediction tools for risk assessment is whether genome sequence data are available for nontarget organisms. Nevertheless, in silico predictions are a prerequisite and critical requirement for developing and rational design of dsRNA-based pesticides. In addition, bioinformatics may help select adequate test species for nontarget organism toxicology and risk assessment (as suggested in OECD, 2020).

In addition to noticeable off-target effects based on sequence similarities, other sequence-unspecific (noncanonical) adverse effects can occur. Given that RNAi has evolved as an antiviral defense mechanism in the plant (Waterhouse et al., 2001) and mammalian innate immunity (Maillard et al., 2013; Poirier et al., 2021), it is unsurprising that dsRNA is a potent inducer of inflammatory responses through receptors (i.e., Toll-like receptor 3) that sense dsRNA (a byproduct of viral replication) (Lin et al., 2008). Interestingly, researchers have speculated about the presence of a dsRNA recognition receptor that can sense viral dsRNAs for plants as well (Niehl and Heinlein, 2019); indeed, dsRNA has been shown to induce plants' pattern-triggered immune signaling (Niehl et al., 2016). However, spray application of dsRNA (designed to target fungal *CYP51* genes) does not induce pattern-triggered immunity in barley, and the spray-induced gene silencing mechanism does not rely on the activation of canonical defense pathways (Koch et al., 2016). This finding

is relevant when considering the fitness cost and thus yield, following foliar dsRNA sprays.

Another possible unintentional adverse effect is the saturation and suppression of endogenous RNAi machinery, observed after applying high doses of exogenous RNAs to mammalian cell cultures (Grimm et al., 2006). However, optimizing doses can easily minimize the risk of oversaturation, and dsRNA will probably never reach such critical levels when applied as a dsRNA-based agricultural pesticide (OECD, 2020). Overall, the possibility that sequence-unrelated adverse effects might occur cannot be excluded, clearly demonstrating a fundamental need for biological assessments and empirical testing to capture the relevant activity spectrum of an RNAi-based pesticide.

Potential risks to nontarget organisms, including humans

A key factor in potential RNA-based pesticide risks and assessment is whether the nontarget organism will be affected when exposed to the application. It applies to users, consumers, and any other (terrestrial, aquatic, avian) nontarget organisms in the (agro-) ecosystem. For RNA-based pesticide application risks to users and consumers, oral, dermal, and inhalation/respiratory exposure is probable. To assess whether such exposure would inevitably lead to human health risks, different working groups have begun to evaluate the knowledge gained from substantial and extensive research conducted on medical applications of dsRNA (EFSA, 2019; OECD, 2020).

The major challenge in the development of oligonucleotide-based therapies to treat human diseases is the limited bioavailability of dsRNA due to the degrading enzymatic and nonenzymatic environment (saliva, stomach, gut) and natural barriers (physical, biochemical, enzymatic) present in most mammals (Nogrady, 2019; Paunovska et al., 2022). For example, it is known that native (unmodified) dsRNA undergoes rapid clearance from circulation (Hu et al., 2020). Notably, almost all siRNA-based drugs administered into the bloodstream tend to accumulate in the liver (Eisenstein, 2006); consequently, RNAi companies have turned their focus to conditions that could be treated by targeting hepatocytes, with the first product, Patisiran (ONPATTRO), approved in 2018 for the treatment of hereditary transthyretin amyloidosis (Hoy, 2018). However, chemical modification is necessary to protect dsRNA from degradation by Ribonucleases and ensure uptake over numerous barriers, including physiological and biochemical barriers (Whitehead et al., 2009). Given these circumstances, the European Food Safety Authority (EFSA) food and feed risk assessment of RNAi-based GM plants (in the case of MON 87411) concluded that "the stability of plant miRNAs under gastrointestinal conditions in vitro, ex vivo or in vivo is low. … the percentage of surviving miRNAs in the gastrointestinal tract in the best-case scenario is around $\approx 1\%$,…" (Dávalos et al., 2019, EFSA Supporting publications). In addition, the OECD working paper concluded that "when metabolism and barriers are considered, it is extremely unlikely that oral ingestion of naked/unformulated dsRNAs will reach mammalian

cells in sufficient quantities to mediate any RNAi effects" (OECD, 2020). However, this preliminary conclusion related to the use of native dsRNAs must be revised if dsRNAs are to be chemically modified or formulated (e.g., via complexing, encapsulation) to increase their stability (Mitter et al., 2017), facilitate cellular uptake (Schwartz et al., 2020), or mediate endosomal escape (Lee et al., 2021). Many such formulations and nanoformulations have been developed to promote the rapid lab-to-field transition of RNA sprays (see more in Rank and Koch, 2021) but will require case-by-case risk assessment by registrants seeking market approval. The medical applications of RNAi therapeutics have yielded data that aids in RNAi-based pesticide risk assessment for mammals, including humans. Mammalian digestive systems and barriers may prevent the activity of ingested dsRNA and therefore limit the potential for adverse effects.

Based on the previous risk assessment of DvSnf7 dsRNA (MON 87411), the United States Environmental Protection Agency (US EPA) stated that nonmammalian vertebrates are expected to have similar digestive effects as mammals (USEPA, 2016). However, these assumptions did not and will not free registrants from testing nontarget organisms. For example, risk assessment of the DvSnf7 dsRNA expressed in corn included bioassays with numerous nontarget species (Table; detailed in USEPA, 2016). The finding that the functionality of RNAi machinery significantly varies among different organisms (Meister and Tuschl, 2004) demonstrates the need for testing to identify risks. Additionally, fundamental mechanistic knowledge to gain insight into dsRNA uptake, transport, and processing is urgently required to determine potential off-target risks. Researchers extrapolated knowledge gained from basic research using model organisms to compensate for the lack of species-specific information. Notably, this research revealed substantial variation in responsiveness to exogenous RNAs among insect orders (Zotti and Smagghe, 2015) and even among related species and individuals of the same species depending on factors including, for example, life stages (Mehlhorn et al., 2020; Pallis et al., 2022). For example, Diptera (flies and mosquitoes), Hemiptera (aphids, hoppers, stinkbugs), and Lepidoptera (moths and butterflies) show great variability compared to Coleoptera species such as corn rootworms and Colorado potato beetles (for an excellent overview, see Cooper et al., 2019; Christiaens et al., 2020). While these results underline the enormous natural variation in how organisms cope with exogenous RNA and usage of RNAi machinery, they reveal uncertainties in understanding RNAi technologies; therefore, ongoing basic research for risk mitigation and to exploit RNAi mechanisms more efficiently is needed.

Environmental fate and the risk of exposure

Another crucial factor that must be considered regarding risk identification and assessment is whether, or at least to what extent, a nontarget organism will be exposed to applied RNA pesticides. Application methods and product formulation of RNA-based pesticides may vary depending on the crop or plant species to which they are applied and the lifestyle or behavior of the target pathogens and pests. In

principle, the same application methods for conventional chemical pesticides may be used, including sprays on leaves, flowers, or plant products (e.g., seeds and fruits); soil drenches, and seed treatments. In addition, uses in the field as well as in greenhouses or crop storage facilities are feasible. Thus far, most studies have demonstrated the effectiveness of RNA-based biopesticides in controlled lab environments, focusing on spray application on leaves (Dalakouras et al., 2016; Koch et al., 2016, 2019; Höfle et al., 2020; Werner et al., 2020) and spotted application on fruits, vegetables, ornamentals (Wang et al., 2016; Qiao et al., 2021), coleoptiles (Song et al., 2018), seedlings (Mitter et al., 2017; Biedenkopf et al., 2020), and flowers (Willow et al., 2020, 2021; see more in Rank and Koch, 2021). However, the same exposure routes must be considered because dsRNA-based pesticides may be applied like conventional pesticides.

Exposure risks strongly depend on the environmental fate of pesticides; understanding the routes by which pesticides are distributed and studying pesticides' stability and persistence in the environment is thus essential to identify potential risks for nontarget organisms. The latest efforts to facilitate the lab-to-field transfer of RNAi-based pesticides have generated conflicts of interest. On the one hand, dsRNA and siRNA must be stabilized to withstand harmful environmental influences (e.g., UV, rain, microbes); on the other hand, they should not be allowed to accumulate in the soil, in water, or along food chains (Rank and Koch, 2021). Therefore, balancing stabilizing measures with guaranteed biodegradability is a great challenge. Native dsRNA (also known as naked dsRNA; DvSnf7 dsRNA) without stabilizing formulations has been shown to rapidly degrade in soils by studies following methods and models developed initially by regulatory agencies to estimate environmental concentrations of chemical pesticides (Dubelman et al., 2014; Parker et al., 2019; Bachman et al., 2020). Based on the data available at the time of the study, researchers concluded that "once RNA reaches the soil, it is unlikely to persist or accumulate" (OECD, 2020). Similar results were obtained from the measurement of dsRNA degradation in aerobic water-sediment systems (Albright et al., 2017; Fischer et al., 2017), leading to the conclusion that "dsRNA-based agricultural products are unlikely to persist in aquatic environments" (OECD, 2020). Although these preliminary conclusions hold true, further data from a broader range of locations would yield more robust results.

Other mechanisms by which dsRNA and siRNA may be dispersed throughout the environment relevant to risk assessment are spray drifts, physical movement (e.g., via insect pollinators), and uptake by roots of nontarget plants (USEPA, 2013). Physical movement in a lively local ecosystem may contribute to the spread of unpredictable amounts of dsRNA, with unknown results. Therefore, it was suggested that "as for other pesticides, regulatory consideration of edge-of-field exposure could be considered a worst-case for off-site exposure" (OECD, 2020). However, in addition to stability, persistence, and off-site movement, there are other essential aspects of the application that must be evaluated on a case-by-case and product-by-product basis in a risk assessment, such as application method (i.e., spray, drench, sprinkle), timepoint (e.g., seasonal), concentration, strategy (e.g., ingredient combination),

and frequency (e.g., multiple dosing). Moreover, testing has thus far been limited to laboratories; whether this is sufficient to simulate or fully reflect the fate of a dsRNA or siRNA applied directly to the environment remains uncertain. However, because organisms are built from RNA and DNA, nucleic acids have long been safely consumed by humans and other vertebrates (OECD, 2020); the question is whether the additional dsRNA or its degradation molecules or products in agriculture will pose risks to nontarget organisms.

What can we learn from the approval of the first GM-based RNAi pesticide (MON 87411)?

The corn SmartStax PRO, developed by Monsanto, contains multiple plant-incorporated protectants. In addition to several proteins derived from *Bacillus thuringiensis* (*Bt*; Cryl A.105, Cry2Ab2, CrylF, Cry3Bbl, Cry34Abl, and Cry35Ab1) for control of lepidopteran and coleopteran pests, the corn also expresses the 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 and the phosphinothricin acetyl transferase protein from *Streptomyces viridochromogenes,* which confer tolerance to glyphosate and glufosinate-ammonium herbicides. Additionally, SmartStax PRO corn expresses a 240 bp DvSnf7-dsRNA (MON 87411) designed to match the sequence of the *Snf7* gene in western corn rootworm (*Diabrotica virgifera virgifera,* WCR) (Head et al., 2017). Until 2016, MON 87411 and the DvSnf7 dsRNA that it expresses were only registered for seed increase, and use was limited to 2 years (USEPA, 2015).

Because of uncertainties related to dsRNA-based pesticides, the US EPA required additional ecological risk assessment data to support full commercial registration (USEPA, 2016). As a baseline, the existing US EPA risk assessment framework for biochemical pesticides and plant-expressed insecticidal substances (developed primarily based on experience with *Bt*-derived Cry proteins; e.g., see USEPA, 2010) was conducted following a tiered testing scheme (Tiers I–IV): "Tier-I studies are designed to be simplified and to estimate of [sic] hazard to several nontarget taxa under 'worst-case' exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed" (40 CFR Part 158). However, in 2014, the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommended consideration of the following questions for human health and ecological risk assessments of RNAi-based products (FIFRA SAP, 2014): (1) Is the dsRNA capable of overcoming natural defenses that could prevent entry into the human body? (2) If so, would the dsRNA be able to find and trigger a reaction that interferes with cellular function? (3) Where would the dsRNA be found in the environment, and how long would it persist? (4) Assuming environmental persistence, which nontarget organisms would be exposed to it? (5) Assuming exposure, would the dsRNA enter the organism, find a target gene, and interfere with a key cellular process?

Based on the results (summarized in the Table; for details, see USEPA, 2016) the US EPA proposed a registration decision for MON 87411: " … since the proposed MON 87411 commercial use products have met the required criteria, the Agency is proposing to grant conditional registrations under FIFRA section 3(c)(7)(A). The proposed registrations will be time-limited to 5 years" (USEPA, 2016).

Regarding the questions raised by the FIFRA SAP (2014), the EPA responded with the following information and based on that, "Applicability for evaluation of exogenously applied dsRNAs" could be estimated (see Table 8.1):

Considering the registration of MON 87411 as appropriate for assessing dsRNA-based pesticides in the United States, regulatory agencies, risk assessors, and applicants must consider some fundamental differences between GM-based endogenous expression of dsRNA and GM-free exogenous (foliar) application of dsRNA. In general, it is expected that external application, especially spray application, may cause a relatively high potential for drift, runoff, or movement to other environmental compartments (Mendelsohn et al., 2020). In contrast, the environmental fate of plant-expressed dsRNA largely depends on its expression levels within different plant tissues and organs (e.g., leaf, pollen, grain) and degradation of the incorporated pesticidal substance within the environment. Given the assumption that corn tissue, in the case of MON 87411, represents the primary source of exposure to terrestrial nontarget organisms, research must first elucidate the routes taken by exogenously applied RNA-based pesticides. So far, uptake and systemic transport to other plant tissues, such as floral tissue, is undetermined. Although several studies have reported the foliar uptake of exogenously applied dsRNA followed by systemic translocation (Koch et al., 2016; Konakalla et al., 2016; Gogoi et al., 2017; Kaldis et al., 2018; Song et al., 2018; Biedenkopf et al., 2020), the OECD working paper concluded that "there is no convincing evidence to date for uptake and systemic transport from application of unformulated dsRNA to intact tissue in the absence of delivery approaches" (OECD, 2020). This conclusion might affect decisions related to risk assessment, as it indicates that testing nontarget plants if the cuticle of plants acts as an impermeable barrier will not be required. Contradictory to this preliminary conclusion, researchers have shown that SIGS-associated RNAs translocated from leaves, where dsRNA was applied, to barley roots, where they inhibited the fungal infection of roots (Biedenkopf et al., 2020). Notably, unformulated dsRNAs were applied on seedlings with an unspecific delivery method (i.e., no abrasion, no high pressure). Therefore, we cannot exclude the possibility that testing of nontarget plants and oral dietary testing (food/feed) would be required as long as uncertainties associated with routes and exposure levels persist.

More problematic or unforeseeable if dsRNA is applied by foliar spray than if transgenically expressed is off-site movement. While the environmental fate of transgene-derived DvSnf7 dsRNA in soil and water relies on the off-site movement of pollen or exudation and was thus determined to be negligible (USEPA, 2017), exposure of terrestrial and aquatic environments via drift and runoff of sprayed dsRNA is considered to be more likely, but, in general, dsRNA is subject to the same abiotic and biotic degradation processes as conventional, chemical, and

Table 8.1 Summary of data submitted for MON 87411 and their applicability for the evaluation of dsRNA-based pesticides.

Guideline nr tier I	Data requirement	Species	Applicability for evaluation of exogenously applied dsRNAs[b]
885.4050	Avian acute oral toxicity	*Gallus domesticus*[a]	Unlikely
850.2100[a]		*Colinus virginianus*	Unlikely
885.4100	Avian inhalation toxicity/ pathogenicity	Not required	Unlikely
885.4150	Wild mammal toxicity/ pathogenicity	Mice, rats	Applicable
885.4200	Freshwater fish toxicity/ pathogenicity	*Ictalurus punctatus*	Unlikely (argument: rapid degradation in water)
885.4240	Freshwater invertebrate toxicity/pathogenicity	Not tested	Unlikely (argument: rapid degradation in water)
885.4280	Estuarine and marine fish testing estuarine and marine invertebrate testing	Not required	Unlikely (argument: rapid degradation in water)
885.4300	Nontarget plant testing	Not required	Uncertain (argument: uptake and systemic transport cannot be excluded)
885.4340	Nontarget insect testing	*Coleomegilla maculata*	Applicable
		Pedibus foviolatus	Applicable
		Orius insidiosus	Applicable
		Poecilus chalcites	Applicable
		Chryoperla carnea	Applicable
		Aleochara bilineata	Applicable
		Folsomia candida	Applicable
885.4380	Honeybee testing	*Apis mellifera*	Applicable
850.6200	Earthworm subchronic toxicity testing	*Eisenia andrei*[a]	Applicable
885.5200	Expression in a terrestrial environment	Soil fate and degradation	Applicable (or not required once convincing data exist)
850.2500	Field testing for terrestrial wildlife	Arthropod abundance[a]	Applicable
	Bioinformatic analysis	23 nontarget organisms[a]	Applicable/Required

[a] *Modified (e.g., extending exposure and observation period).*
[b] *Classification: supplemental.*

biological pesticides (OECD, 2020). So far, as mentioned earlier, existing data support rapid degradation rates in soil and water. For example, the soil/water fate and degradation of DvSnf7 dsRNA were investigated using in vitro transcribed dsRNA (USEPA, 2016) and verified its rapid soil degradation; 90% dissipation took 45−55 h. These data provide a good informational base, but as long as there is a lack of sufficient data for applicants to refer to, they will be required to generate degradation rates of dsRNA in soil and water matrices under aerobic and anaerobic conditions and variable conditions of UV light and pH upon registration approval.

Because exposure for nontarget organisms is probable, how to select representative nontarget organisms for risk assessment testing should be considered. There are no guidelines outlining data requirements for risk assessment of exogenously applied RNAi-based products. In general, the selection of suitable nontarget organisms is based on three prerequisites: (1) sensitivity, meaning that the organism must be sensitive to environmental RNAi; (2) representativeness, meaning that the organism must be representative for valued taxa or functional groups likely to be exposed in the field; and (3) availability and reliability, meaning that suitable life-stages of the test species must be obtainable in sufficient quantity and quality and that validated test protocols must be available that allow consistent detection of adverse effects on ecologically relevant parameters (Romeis and Widmer, 2020). Given these assumptions, the OECD working paper outlined a useful decision tree for the selection of nontarget organisms for empirical testing, which can be summarized as follows:

Does the species with protection goals have any potential for exposure?

NO: Species not suitable for empirical testing.

YES Is the species with protection goals responsive to environmental RNAi?

NO: Species not suitable for empirical testing.

YES Does the species with protection goals have significant bioinformatic alignment?

NO: Species not suitable for empirical testing.

YES Are data available that are relevant to species with protection goals?

NO: No empirical testing needed.

YES Are laboratory test protocols available that can be used for species with protection goals?

NO: Reconsider options for risk assessment.

YES Consider empirical testing.

In addition, De Schutter et al. (2022) provided a valuable overview of potential terrestrial, aquatic, and avian nontarget species for which validated test protocols are available, allowing for risk assessment of dsRNA. However, since the approval of MON 87411, the OECD working paper has provided some initial ideas on how the registration procedure will proceed; research and industry await the first decisions on exogenously applied dsRNA-based pesticides seeking to enter the market.

Related to this, Greenlight Biosciences and Syngenta have reported promising early results from field trials of dsRNA-treated plants demonstrating control of the Colorado potato beetle (Fig. 8.2) (Bramlett et al., 2020; Rodrigues et al.,

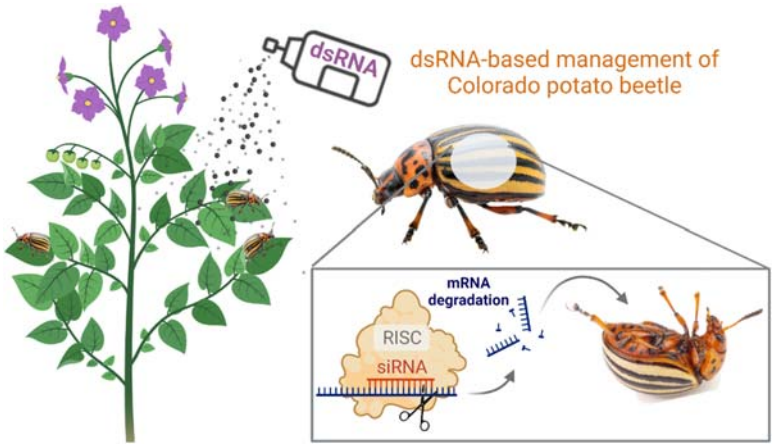

FIGURE 8.2

dsRNA spray-based management of *Leptinotarsa decemlineata*.

Created with BioRender.com.

2021). The most promising candidate in the pipeline is *ledprona* (US Patent No. 11142768), a sprayable dsRNA-based insecticidal active ingredient developed by Greenlight Bioscience. The 490 bp dsRNA was designed to target the proteasome subunit beta 5 (dsPSMB5) synthesis by the Colorado potato beetle, *Leptinotarsa decemlineata* (Rodrigues et al., 2021) (Fig. 8.2). Greenlight Bioscience applied to the US EPA in June 2021, requesting an experimental use permit for *ledprona* to conduct large-scale field trials on potatoes using multiple commercial application methods and equipment and gather data on efficacy and crop safety to understand better how dsPSMB5 performs. The proposed experimental program would begin on April 1, 2022, and continue until April 1, 2023; a total of 3700 g of dsPSMB5 would be applied to 200 acres of potatoes in 11 US states (Idaho, Maine, Michigan, Minnesota, New York, North Carolina, North Dakota, Oregon, Virginia, Washington, and Wisconsin). Notably, an experimental use permit had not yet been granted at the time of writing this article. However, recent results have shown that exposure to *ledprona* increased Colorado potato beetle mortality and decreased foliage consumption in all four instars and adult beetles in both detached leaf bioassays and field trials (Pallis et al., 2022). Defoliation and yield protection levels were comparable to those provided by spinosad and chlorantraniliprole. Notably, formulated *ledprona* was most effective when applied at 7-day intervals for three consecutive weeks. This study provided valuable information on product formulation (aqueous-based soluble concentrate), applied concentration (4.7–9.9 g ha^{-1}, which is less than 10 times that of traditional chemical pesticides), application rate of the exogenously applied dsRNA active ingredient (weekly), application method (pressure of 207 kPa, liquid output of 188 L ha^{-1}, and travel speed of 0.8 km h^{-1}), and the number of applications (three) required for environmental risk assessment (Pallis et al., 2022).

Interestingly, dsPSMB5 was observed to act relatively slowly (7–8 days to reach 99% mortality) compared with traditional chemical pesticides (Pallis et al., 2022). Consistent with this observation, it was previously reported and assumed that mortality onset would be later under RNAi-based pesticides (Pallis et al., 2022 and references cited therein). Whether this is a disadvantage of exogenously applied RNA-based pesticides per se, because pests may cause severe damage before succumbing to their lethal effects remains to be verified. Given the slow-acting nature of dsRNA-based pesticides, researchers, including Bachman et al. (2016), have suggested adapting acute nontarget study protocols by extending the evaluation period.

As the first GM RNAi plant, MON 87411 was assessed under the existing US regulatory framework. Currently, dsRNA-based pesticides are considered chemical plant protection products in the EU and are regulated as such (see more in Dietz-Pfeilstetter et al., 2021). However, because none of the existing EU regulation categories is a perfect fit for assessing dsRNA-based pesticides, the consensus is that appropriate, modified safety evaluations and adapted approval and authorization procedures are required. However, procedures for classifying and authorizing dsRNA-based pesticides may vary according to current US and EU requirements and regulations adopted in the future (detailed in recent reviews: Dietz-Pfeilstetter et al., 2021; De Schutter et al., 2022).

Conclusion and perspectives

Complex and multi-layered challenges must be overcome to make exogenous RNA applications a realistic technique in plant production. Despite Greenlight Bioscience's invention of *ledprona* and the numerous proof-of-concept studies that illustrate the potential of RNA active ingredients in plant protection, data regarding field trials of dsRNA remain somewhat limited. Currently, scientists are conducting extensive research to develop solutions that will guarantee the stability, selectivity, and broad applicability of dsRNA-based pesticides to improve overall field performance. Additionally, policymakers and regulatory authorities are seeking more information on the diversity and functionality of RNAi mechanisms through cooperation with relevant experts from academia and industry. This cooperation will facilitate the development of appropriate approaches to the risk assessment of novel dsRNA-based products.

For optimal effectiveness, RNAs should be as stable and durable as possible and capable of degrading rapidly without leaving environmentally harmful residues. To meet these requirements, considerable progress has been made to further develop dsRNA-based pesticides. For example, the development of nanotechnology-based formulations is rapidly advancing. Crop protection based on RNA biopesticides can also benefit from developments in this field, especially because many of the formulations already in use have been inspired by their medical applications. However, the behavior and fate of (nano)formulated RNAs in the environment still must be investigated and evaluated. Furthermore, more research on the persistence and

biodegradability of sprayed RNAs for developing dsRNA-specific risk assessments is urgently required. This contradiction calls for innovative solutions, some of which may be found in the fusion or transfer of various technologies.

The need for novel, powerful agricultural tools to replace conventional chemical pesticides and meet society and politicians' increasing demands for sustainable plant production has raised several questions. Concerning their handling by potential users, (1) when, how often and in what quantities must the RNAs be applied? (2) Are there any special requirements for handling and storing active RNA agents? (3) What will RNA biopesticides cost? (4) For which cultures and pathogens will active agents be available? Further questions have arisen from environmentalists and consumers: (5) Is the consumption of RNA-treated plants and derived commodities safe? (6) Will RNA-treated food be more expensive than food produced using traditional pesticides? (7) What are the effects of spraying RNAs on local ecosystems? (8) Will RNAs accumulate in the soil or in the water? The following questions are relevant from an entrepreneurial perspective: (9) How much will it cost to approve RNA active agents? (10) How will these be regulated? (11) Is the development of resistances to be expected? (12) Will RNA agents be accepted by the public?

We have already addressed some of these questions and concerns in this article. One of the most pressing questions is whether and what risks will arise from consuming foods treated with RNAs or products derived from them. In brief, the absorbed RNAs have gene-regulatory potential because they bind complementary mRNAs and interfere with their translation into a functional protein. In this context, a recently published review article collected and assessed the evidence for and against the transmission of food-derived miRNAs and their gene regulatory function from plants, meat, and milk (Mar-aguilar et al., 2020; del Pozo-Acebo et al., 2021). The authors stressed that the transmission of miRNAs from food to the blood had not been conclusively or convincingly demonstrated. The leading cause for the controversial assessment is nonreproducible data. The EFSA currently considers spraying RNA-based pesticides safe (Dávalos et al., 2019). The risk of dysfunctional gene expression in humans caused by the consumption of RNAi products is low. The decisive argument was that, following oral uptake of RNA, too many biological and physical barriers would have to be overcome; consequently, the ingested RNA would likely be degraded before binding a potential off-target mRNA (Schiemann et al., 2019; Kleter, 2020). However, the controversy concerning human health risks may continue and will probably accelerate if the use of RNA-based pesticides attracts public attention. Therefore, participants of the 2019 OECD Conference on RNAi-based pesticides stressed the need to communicate about this new technology to the public (Mendelsohn et al., 2020). A major concern was that the people might misinterpret RNA pesticides as a "new genomic technique" raising questions about the best way to market RNAi technology so that it is not incorrectly perceived as genetic modification (Mendelsohn et al., 2020). Overall, public communication will be challenging but should appeal to common sense and aim to reduce skepticism through transparency, awareness campaigns, and convincing marketing strategies.

Another urgent question is whether the use of RNA-based pesticides will hold true to its promise of overcoming resistance problems. A preliminary study was published in 2018 by Monsanto (since acquired by Bayer CropScience) (Khajuria et al., 2018), which aimed to anticipate possible resistance mechanisms against dsRNAs in insects. A population of Western corn rootworms, *Diabrotica virgifera virgifera*, was explicitly selected for dsRNA (DvSnf7) resistance. Resistance manifested in the insects' inability to absorb the maize-expressed DvSnf7 dsRNA through the intestine. This inability was sequence-independent and was also evident after the injection of dsRNA into the hemolymph. The findings of this study formed the basis for the development of the GM corn SmartStax PRO, in which resistance development is counteracted by the combination of different active agents with different modes of action (as discussed earlier). In addition, a follow-up study concluded that resistance is much more likely to arise as a result of exogenous RNA application than GM-based exploitation of RNAi (Mishra et al., 2021). However, the extent to which these artificially selected resistances reflect natural processes by which resistance might evolve remains unclear. Likewise, the extent to which these scenarios would apply to other harmful insects that develop resistance to active agents less quickly than the corn rootworm (Meinke et al., 2021) and the potato beetle (Alyokhin et al., 2008) also remains unknown.

Despite these essential early findings, many questions remain unanswered, requiring ongoing research to be carried out in close cooperation with the responsible authorities and organizations. Additionally, the advantages of this technology over existing methods must be clearly emphasized to the public. One strong argument for using RNA (sequence-based) active ingredients lies in their high selectivity and potential to respond very quickly and precisely to emerging or suddenly occurring pests and pest epidemics. The importance of this feature was made clear by the emergence of the SARS-CoV-2 virus in 2020; a pandemic of similar severity and consequence could occur among crops. However, the advantages of pesticides that rely on naturally occurring RNAi will only prevail if they are subjected to a modified and shortened approval procedure. Although this possibility exists and has been granted by the EU (EC, 2009), there are currently no specific guidelines for assessing RNA-based active agents (Schenkel and Gathmann, 2021). *Ledprona* may pave the way for the future approval of further RNA biopesticides (Rodrigues et al., 2021), though risk assessment will initially take place on a case-by-case basis.

RNAi is a fast-growing technology, and the first products using it will be commercially available in the next few years and will likely be widely used. In the future, it will be important to further deepen knowledge of the molecular mechanisms underlying exogenous RNA applications and to continuously integrate new findings into further developments and evidence-based risk assessments. For these reasons, scientists see themselves as responsible for constantly evaluating the safety of dsRNA-based products.

References

Albright III, V.C., Wong, C.R., Hellmich, R.L., Coats, J.R., 2017. Dissipation of double-stranded RNA in aquatic microcosms. Environmental Toxicology and Chemistry 36, 1249−1253. https://doi.org/10.1002/etc.3648.

Alyokhin, A., Baker, M., Mota-Sanchez, D., Dively, G., Grafius, E., 2008. Colorado potato beetle resistance to insecticides. American Journal of Potato Research 85, 395−413. https://doi.org/10.1007/s12230-008-9052-0.

Bachman, P.M., Huizinga, K.M., Jensen, P.D., Mueller, G., Tan, J., Uffman, J.P., et al., 2016. Ecological risk assessment for DvSnf7 RNA: A plant-incorporated protectant with targeted activity against western corn rootworm. Regulatory Toxicology and Pharmacology 81, 77−88. https://doi.org/10.1016/j.yrtph.2016.08.001.

Bachman, P., Fischer, J., Song, Z., Urbanczyk-Wochniak, E., Watson, G., 2020. Environmental fate and dissipation of applied dsRNA in soil, aquatic systems, and plants. Frontiers in Plant Science 11, 21.

Biedenkopf, D., Will, T., Knauer, T., Jelonek, L., Furch, A.C.U., Busche, T., et al., 2020. Systemic spreading of exogenous applied RNA biopesticides in the crop plant *Hordeum vulgare*. ExRNA 2. https://doi.org/10.1186/s41544-020-00052-3.

Bramlett, M., Plaetinck, G., Maienfisch, P., 2020. RNA-based biocontrols—a new paradigm in crop protection. Engineering 6, 522−527. https://doi.org/10.1016/j.eng.2019.09.008.

CBD/WG, 2020. Report of the Open-Ended Working Group on the Post-2020 Global Biodiversity Framework on its Third Meeting. September 2021 Available at: WG2020-03 - Documents (cbd.int).

Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M.R., Urru, I., et al., 2018. Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. EFSA Supporting Publications 15, 1424E. https://doi.org/10.2903/sp.efsa.2018.EN-1424.

Christiaens, O., Whyard, S., Vélez, A.M., Smagghe, G., 2020. Double-stranded RNA technology to control insect pests: current status and challenges. Frontiers in Plant Science 11, 451. Available at: https://www.frontiersin.org/article/10.3389/fpls.2020.00451.

Cooper, A.M.W., Silver, K., Zhang, J., Park, Y., Zhu, K.Y., 2019. Molecular mechanisms influencing efficiency of RNA interference in insects. Pest Management Science 75, 18−28. https://doi.org/10.1002/ps.5126.

Dalakouras, A., Ganopoulos, I., 2021. Induction of promoter DNA methylation upon high-pressure spraying of double-stranded RNA in plants. Agronomy 11. https://doi.org/10.3390/agronomy11040789.

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M.L., Papadopoulou, K., 2020. Genetically modified organism-free RNA interference: exogenous application of RNA molecules in Plants. Plant Physiology 182, 38−50. https://doi.org/10.1104/pp.19.00570.

Dalakouras, A., Wassenegger, M., McMillan, J.N., Cardoza, V., Maegele, I., Dadami, E., et al., 2016. Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. Frontiers in Plant Science 7, 1327. Available at: https://www.frontiersin.org/article/10.3389/fpls.2016.01327.

Dávalos, A., Henriques, R., Latasa, M.J., Laparra, M., Coca, M., 2019. Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of

ncRNA-based genetically modified plants for food and feed. EFSA Supporting Publications 16, 1688E. https://doi.org/10.2903/sp.efsa.2019.EN-1688.

De Schutter, K., Taning, C.N.T., Van Daele, L., Van Damme, E.J.M., Dubruel, P., Smagghe, G., 2022. RNAi-based biocontrol products: market status, regulatory aspects, and risk assessment. Frontiers in Insect Science 1, 22. Available at: https://www.frontiersin.org/article/10.3389/finsc.2021.818037.

del Pozo-Acebo, L., López de las Hazas, M.-C., Margollés, A., Dávalos, A., García-Ruiz, A., 2021. Eating microRNAs: pharmacological opportunities for cross-kingdom regulation and implications in host gene and gut microbiota modulation. British Journal of Pharmacology 178, 2218−2245. https://doi.org/10.1111/bph.15421.

Dietz-Pfeilstetter, A., Mendelsohn, M., Gathmann, A., Klinkenbuß, D., 2021. Considerations and regulatory approaches in the USA and in the EU for dsRNA-based externally applied pesticides for plant protection. Frontiers in Plant Science. 12. https://doi.org/10.3389/fpls.2021.682387.

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al., 2014. Environmental fate of double-stranded RNA in agricultural soils. PLoS One 9, e93155. Available at: https://doi.org/10.1371/journal.pone.0093155.

EC, 2009. Regulation (EC) no. 1107/2009 of the European parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union L 309, 1−50.

EC, 2019. The European Green Deal; Communication from the Commission to the European Parliament, the European Council, the Council. the European Economic and Social Committee and the Committee of the Regions (COM(2019) 640 final).

EC, 2018. European Commission, Directorate-General for Research and Innovation, A Sustainable Bioeconomy for Europe: Strengthening the Connection between Economy, Society and the Environment: Updated Bioeconomy Strategy. Publications Office, p. 2018. https://data.europa.eu/doi/10.2777/478385.

EC, 2020. Biodiversity Strategy for 2030 Bringing Nature back into Our Lives; Communication from the Commission to the European Parliament, the Council. the European Economic and Social Committee and the Committee of the Regions EU (COM/2020/380 final).

EFSA, 2019. Panel on Genetically Modified Organisms (GMO), Naegeli H, Bresson JL et al (2019) Assessment of genetically modified maize Mon 87427× Mon 89034× MIR 162× Mon 87411 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2017-144). EFSA Journal 17, e05848.

Eisenstein, M., 2006. Pharma's RNA roller coaster. Nature 574, S4−S6.

FAO, 2018. Transforming Food and Agriculture to Achieve the SDGs 20 Interconnected Actions to Guide Decision-Makers. FAO, Rome. Available at: i9900en.pdf.(fao.org).

FIFRA SAP, 2014. SAP Minutes No. 2014-02: a set of scientific issues being considered by the Environmental Protection Agency Regarding RNAi technology program formulation for human health and ecological risk assessment. In: FIFRA SAP Meeting held January 28, 2014, Arlington, Virginia, USA. Available at: https://www.epa.gov/sites/default/files/2015-06/documents/012814minutes.pdf.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806−811. https://doi.org/10.1038/35888.

Fischer, J.R., Zapata, F., Dubelman, S., Mueller, G.M., Uffman, J.P., Jiang, C., et al., 2017. Aquatic fate of a double-stranded RNA in a sediment−water system following an over-water application. Environmental Toxicology and Chemistry 36, 727−734. https://doi.org/10.1002/etc.3585.

Gaffar, F.Y., Koch, A., 2019. Catch me if you can! RNA silencing-based improvement of antiviral plant immunity. Viruses 11. https://doi.org/10.3390/v11070673.

Gogoi, A., Sarmah, N., Kaldis, A., Perdikis, D., Voloudakis, A., 2017. Plant insects and mites uptake double-stranded RNA upon its exogenous application on tomato leaves. Planta 246, 1233−1241. https://doi.org/10.1007/s00425-017-2776-7.

Grimm, D., Streetz, K.L., Jopling, C.L., Storm, T.A., Pandey, K., Davis, C.R., et al., 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441, 537−541. https://doi.org/10.1038/nature04791.

Head, G.P., Carroll, M.W., Evans, S.P., Rule, D.M., Willse, A.R., Clark, T.L., et al., 2017. Evaluation of SmartStax and SmartStax PRO maize against western corn rootworm and northern corn rootworm: efficacy and resistance management. Pest Management Science 73, 1883−1899. https://doi.org/10.1002/ps.4554.

Hendrix, B., Hoffer, P., Sanders, R., Schwartz, S., Zheng, W., Eads, B., et al., 2021. Systemic GFP silencing is associated with high transgene expression in *Nicotiana benthamiana*. PLOS One 1−15. https://doi.org/10.1371/journal.pone.0245422.

Höfle, L., Biedenkopf, D., Werner, B.T., Shrestha, A., Jelonek, L., Koch, A., 2020. Study on the efficiency of dsRNAs with increasing length in RNA-based silencing of the Fusarium CYP51 genes. RNA Biology 17, 463−473. https://doi.org/10.1080/15476286.2019.1700033.

Hoy, S.M., 2018. Patisiran: first global approval. Drugs 78, 1625−1631. https://doi.org/10.1007/s40265-018-0983-6.

Hu, B., Zhong, L., Weng, Y., Peng, L., Huang, Y., Zhao, Y., et al., 2020. Therapeutic siRNA: state of the art. Signal Transduction and Targeted Therapy 5, 101. https://doi.org/10.1038/s41392-020-0207-x.

ISAAA, 2019. Global Status of Commercialized Biotech/GMcrops in 2019: Biotech crops Drive Socio-Economic Development and Sustainable Environment in the New Frontier. ISAAA, Ithaca, NY.

Kaldis, A., Berbati, M., Melita, O., Reppa, C., Holeva, M., Otten, P., et al., 2018. Exogenously applied dsRNA molecules deriving from the Zucchini yellow mosaic virus (ZYMV) genome move systemically and protect cucurbits against. ZYMV Molecular Plant Pathology 19, 883−895. https://doi.org/10.1111/mpp.12572.

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W., Pleau, M., et al., 2018. Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. PLoS One 13, e0197059.

Kleter, G.A., 2020. Food safety assessment of crops engineered with RNA interference and other methods to modulate expression of endogenous and plant pest genes. Pest Management Science 76, 3333−3339. https://doi.org/10.1002/ps.5957.

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al., 2016. 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 Pathogence 12, 1−22. https://doi.org/10.1371/journal.ppat.1005901.

Koch, A., Höfle, L., Werner, B.T., Imani, J., Schmidt, A., Jelonek, L., et al., 2019. SIGS vs HIGS: a study on the efficacy of two dsRNA delivery strategies to silence Fusarium

FgCYP51 genes in infected host and non-host plants. Molecular Plant Pathology 20, 1636−1644. https://doi.org/10.1111/mpp.12866.

Koch, A., Kogel, K.H., 2014. New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. Plant Biotechnology Journal 12, 821−831. https://doi.org/10.1111/pbi.12226.

Koch, A., Petschenka, G., 2022. Exogene Anwendung von RNA zur umweltfreundlichen Bekämpfung von Schadinsekten Exogenous application of RNA for the eco-friendly control of insect pests Aktuelle Herausforderungen. Pflanzen- Erhalt der Biodiversität als Ziel 74, 75−84. https://doi.org/10.5073/JfK.2022.03-04.05.

Koch, A., Wassenegger, M., 2021. Host-induced gene silencing − mechanisms and applications. New Phytologist 231, 54−59. https://doi.org/10.1111/nph.17364.

Konakalla, N.C., Kaldis, A., Berbati, M., Masarapu, H., Voloudakis, A.E., 2016. Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. Planta 244, 961−969. https://doi.org/10.1007/s00425-016-2567-6.

Kwon, D.H., Park, J.H., Ashok, P.A., Lee, U., Lee, S.H., 2016. Screening of target genes for RNAi in Tetranychus urticae and RNAi toxicity enhancement by chimeric genes. Pesticide Biochemistry and Physiology 130, 1−7. https://doi.org/10.1016/j.pestbp.2015.11.005.

Lee, J., Sands, I., Zhang, W., Zhou, L., Chen, Y., 2021. DNA-inspired nanomaterials for enhanced endosomal escape. Proceeding of the National Academy of Sciences 118, 3−5. https://doi.org/10.1073/pnas.2104511118/-/DCSupplemental.Published.

Lin, L., Istvan, B., Yan, W., Joseph, S., et al., 2008. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science (80-.) 320, 379−381. https://doi.org/10.1126/science.1155406.

Liu, S., Jaouannet, M., Dempsey, D.A., Imani, J., Coustau, C., Kogel, K.H., 2020. RNA-based technologies for insect control in plant production. Biotechnology Advances 39, 107463. https://doi.org/10.1016/j.biotechadv.2019.107463.

Maillard, P.V., Ciaudo, C., Marchais, A., Li, Y., Jay, F., Ding, S.W., Voinnet, O., 2013. Antiviral RNA interference in mammalian cells. Science (80-.) 342, 235−238. https://doi.org/10.1126/science.1241930.

Mar-aguilar, F., Arreola-triana, A., Mata-cardona, D., Gonzalez-villasana, V., Rodríguez-padilla, C., 2020. Evidence of transfer of miRNAs from the diet to the blood still inconclusive. PeerJ 8, e9567. https://doi.org/10.7717/peerj.9567.

Mehlhorn, S.G., Geibel, S., Bucher, G., Nauen, R., 2020. Profiling of RNAi sensitivity after foliar dsRNA exposure in different European populations of Colorado potato beetle reveals a robust response with minor variability. Pesticide Biochemistry and Physiology 166, 104569. https://doi.org/10.1016/j.pestbp.2020.104569.

Meinke, L.J., Souza, D., Siegfried, B.D., 2021. The use of insecticides to manage the western corn rootworm, *Diabrotica virgifera virgifera*, LeConte: history, field-evolved resistance, and associated mechanisms. Insects 12. https://doi.org/10.3390/insects12020112.

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343−349. https://doi.org/10.1038/nature02873.

Mendelsohn, M.L., Gathmann, A., Kardassi, D., Sachana, M., Hopwood, E.M., Dietz-Pfeilstetter, A., et al., 2020. Summary of discussions from the 2019 OECD conference on RNAi based pesticides. Frontiers in Plant Science 11. Available at: https://www.frontiersin.org/article/10.3389/fpls.2020.00740.

Mishra, S., Dee, J., Moar, W., Beattie, J.D., Baum, J., Dias, N.P., et al., 2021. Selection for high levels of resistance to double - stranded RNA (dsRNA) in Colorado potato beetle (*Leptinotarsa decemlineata* Say) using non - transgenic foliar delivery. Scientific Reports 1−12. https://doi.org/10.1038/s41598-021-85876-1.

Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., et al., 2017. Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. Nature Plants 3. https://doi.org/10.1038/nplants.2016.207.

Niehl, A., Heinlein, M., 2019. Perception of double-stranded RNA in plant antiviral immunity. Molecular Plant Pathology 20, 1203−1210. https://doi.org/10.1111/mpp.12798.

Niehl, A., Wyrsch, I., Boller, T., Heinlein, M., 2016. Double-stranded RNAs induce a pattern-triggered immune signaling pathway in plants. New Phytologist 211, 1008−1019. https://doi.org/10.1111/nph.13944.

Nogrady, B., 2019. The challenge of delivering RNA-interference therapeutics to their target cells. Nature 574, S8−S9.

Nowara, D., Schweizer, P., Gay, A., Lacomme, C., Shaw, J., Ridout, C., et al., 2010. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 22, 3130−3141. https://doi.org/10.1105/tpc.110.077040.

OECD, 2020. Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied Ds-RNA-Based Pesticides Series on Pesticides No. 104 ENV/JM/MONO(2020)26. Available at: https://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2020)26&doclanguage=en.

Pallis, S., Alyokhin, A., Manley, B., Rodrigues, T.B., Buzza, A., Barnes, E., et al., 2022. Toxicity of a novel dsRNA-based insecticide to the Colorado potato beetle in laboratory and field trials. Pest Management Science 78, 3836−3848. https://doi.org/10.1002/ps.6835.

Parker, K.M., Barragán Borrero, V., van Leeuwen, D.M., Lever, M.A., Mateescu, B., Sander, M., 2019. Environmental fate of RNA interference pesticides: adsorption and degradation of double-stranded RNA molecules in agricultural soils. Environmental Science Technology 53, 3027−3036. https://doi.org/10.1021/acs.est.8b05576.

Paunovska, K., Loughrey, D., Dahlman, J.E., 2022. Drug delivery systems for RNA therapeutics. Nature Reviews Genetics 23, 265−280. https://doi.org/10.1038/s41576-021-00439-4.

Poirier, E.Z., Buck, M.D., Chakravarty, P., Carvalho, J., Frederico, B., Cardoso, A., et al., 2021. An isoform of Dicer protects mammalian stem cells against multiple RNA viruses. Science (80-.) 373, 231−236. https://doi.org/10.1126/science.abg2264.

Qiao, L., Lan, C., Capriotti, L., Ah-Fong, A., Nino Sanchez, J., Hamby, R., et al., 2021. Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. Plant Biotechnology Journal 19, 1756−1768. https://doi.org/10.1111/pbi.13589.

Rank, A.P., Koch, A., 2021. Lab-to-Field transition of RNA spray applications − how far are we? Frontiers in Plant Science 12, 2243. Available at: https://www.frontiersin.org/article/10.3389/fpls.2021.755203.

Rodrigues, T.B., Mishra, S.K., Sridharan, K., Barnes, E.R., Alyokhin, A., Tuttle, R., et al., 2021. First sprayable double-stranded RNA-based biopesticide product targets proteasome subunit beta type-5 in Colorado potato beetle (*Leptinotarsa decemlineata*). Frontiers in Plant Science 12. Available at: https://www.frontiersin.org/article/10.3389/fpls.2021.728652.

Romeis, J., Widmer, F., 2020. Assessing the risks of topically applied dsRNA-based products to non-target arthropods. Frontiers in Plant Science 11. Available at: https://www.frontiersin.org/article/10.3389/fpls.2020.00679.

Rosa, C., Kuo, Y.W., Wuriyanghan, H., Falk, B.W., 2018. RNA interference mechanisms and applications in plant pathology. Annual Review of Phytopathology 56, 581−610. https://doi.org/10.1146/annurev-phyto-080417-050044.

Schenkel, W., Gathmann, A., 2021. Regulatory aspects of RNAi in plant production, pp. 154−158. https://doi.org/10.1079/9781789248890.0014.

Schiemann, J., Dietz-Pfeilstetter, A., Hartung, F., Kohl, C., Romeis, J., Sprink, T., 2019. Risk assessment and regulation of plants modified by modern biotechniques: current status and future challenges. Annual Review of Plant Biology 70, 699−726. https://doi.org/10.1146/annurev-arplant-050718-100025.

Schwartz, S.H., Hendrix, B., Hoffer, P., Sanders, R.A., Zheng, W., 2020. Carbon dots for ef fi cient small interfering RNA delivery and gene silencing in plants. Plant Physiology 184, 647−657. https://doi.org/10.1104/pp.20.00733.

Shaffer, L., 2020. RNA-based pesticides aim to get around resistance problems. Proceedings of the National Academy Sciences 117, 32823−32826. https://doi.org/10.1073/pnas.2024033117.

Song, X.-S., Gu, K.-X., Duan, X.-X., Xiao, X.-M., Hou, Y.-P., Duan, Y.-B., et al., 2018. Secondary amplification of siRNA machinery limits the application of spray-induced gene silencing. Molecular Plant Pathology 19, 2543−2560. https://doi.org/10.1111/mpp.12728.

USEPA, 2010. Biopesticides registration action document: *Bacillus thuringiensis* Cry3Bbl protein and the genetic material necessary for its production in Mon 863 Com; *Bacillus thuringiensis* Cry3Bbl protein and the genetic material necessary for its production in Mon 88017 Com. Biopesticides and Pollution Prevention Division, Office of Pesticide Programs. Available at: https://www3.epa.gov/pesticides/chem_search/reg_actions/pip/cry3bb1-brad.pdf.

USEPA, 2013. White Paper on RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment. (Submitted to the FIFRA Science Advisory Panel for Review on Comment. October 29, 2013). Available at: https://www.regulations.gov/document/EPA-HQ-OPP-2013-0485-0011.

USEPA, 2015. Memorandum from S. Borges through C. Wozniak to J. Kausch. Subject: Environmental Risk Assessment for a FIFRA Section 3 Limited Seed Increase Registration 57 of DvSnt7 Double Stranded RNA (dsRNA) and Cry3Bbl *Bacillus thuringiensis* Derived Insecticidal Protein as Expressed in MON 87411 Maize, Dated August 24, 2015.

USEPA, 2016. Environmental Risk Assessment for a FIFRA Section 3 Registration of MON 89034 x TC1507 x MON 87411 x DAS-59122-7 Combined Trait Maize Expressing Cry 1A.105, Cry2Ab2, Cry 1F, Cry3Bb 1, Cry34/35Ab 1 *Bacillus thuringiensis* Derived Insecticidal Protein, and DvSnf7 Double Stranded RNA (dsRNA). Available at: https://www.regulations.gov/document?D=EPA-HQ-OPP-2016-0349-0009.

USEPA, 2017. Proposed registration decision for commercial use corn products containing the DvSnf7 dsRNA plant-incorporated protectant (Event Mon 87411). Available at: https://www.regulations.gov/document?D=EPA-HQ-OPP-2014-0293-0406.

Van Leeuwen, T., Vontas, J., Tsagkarakou, A., Dermauw, W., Tirry, L., 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: A review. Insect Biochemistry and Molecular Biology 40, 563−572. https://doi.org/10.1016/j.ibmb.2010.05.008.

Wagner, D.L., Grames, E.M., Forister, M.L., Berenbaum, M.R., Stopak, D., 2021. Insect decline in the Anthropocene: Death by a thousand cuts. Proceedings of the National Academy of Sciences 118, e2023989118. https://doi.org/10.1073/pnas.2023989118.

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B.P.H.J., Huang, H.-D., Jin, H., 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nature Plants 2, 16151. https://doi.org/10.1038/nplants.2016.151.

Waterhouse, P.M., Wang, M.-B., Lough, T., 2001. Gene silencing as an adaptive defence against viruses. Nature 411, 834−842. https://doi.org/10.1038/35081168.

Werner, B.T., Gaffar, F.Y., Schuemann, J., Biedenkopf, D., Koch, A.M., 2020. RNA-Spray-Mediated silencing of *Fusarium graminearum* AGO and DCL genes improve barley disease resistance. Frontiers in Plant Science 11, 476. Available at: https://www.frontiersin.org/article/10.3389/fpls.2020.00476.

Whitehead, K.A., Langer, R., Anderson, D.G., 2009. Knocking down barriers: advances in siRNA delivery. Nature Reviews Drug Discovery 8, 129−138. https://doi.org/10.1038/nrd2742.

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Nji, C., et al., 2020. First evidence of bud feeding-induced RNAi in a crop pest via exogenous application of dsRNA. Insects 1−8.

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A.I., Taning, C.N.T., et al., 2021. RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. Communications Biology 4, 444. https://doi.org/10.1038/s42003-021-01975-9.

Zhang, H., Cao, Y., Xu, D., Goh, N.S., Demirer, G.S., Cestellos-blanco, S., et al., 2021. Gold-nanocluster-mediated delivery of siRNA to intact plant cells for efficient gene knockdown. Nano Letters 21, 5859−5866. https://doi.org/10.1021/acs.nanolett.1c01792.

Zhang, Y., Mei, S., Wang, Q., 2020. Calcium-siRNA nanocomplexes optimized by bovine serum albumin coating can achieve convenient and efficient siRNA delivery for periodontitis therapy. Journal of Nanomedicine 15, 9241.

Zotti, M., dos Santos, E.A., Cagliari, D., Christiaens, O., Taning, C.N.T., Smagghe, G., 2018. RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. Pest Managment Science 74, 1239−1250. https://doi.org/10.1002/ps.4813.

Zotti, M.J., Smagghe, G., 2015. RNAi technology for insect management and protection of beneficial insects from diseases: lessons, challenges and risk assessments. Neotropical Entomology 44, 197−213. https://doi.org/10.1007/s13744-015-0291-8.

Further reading

OECD, 2019. OECD Conference on RNAi Based Pesticides. OECD Conference on RNAi based Pesticides - OECD.