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RNA Interference in Agriculture: Methods, Applications, and Governance

Biopesticides are one of the many uses of RNA interference to help combat insects such as the Colorado potato beetle—a major pest in potato growing regions. Biopesticides can offer alternatives to chemical-based pesticides that may pose higher potential risks or have reduced effectiveness because of resistance issues. Photo by Tricky_Shark/Shutterstock.

Abstract

RNA interference (RNAi) is a naturally occurring gene silencing mechanism conserved across organisms with a clearly defined cell nucleus (eukaryotes). Gene silencing by RNAi through the degradation of a target messenger RNA (mRNA) has historically been used as a research tool to study the function of genes. Over the past two decades, silencing of vital genes through RNAi has been explored for agricultural applications, including managing plant insect pests and pathogens, improving plant agronomic traits, and increasing consumer desirability of food. Using RNAi for crop protection is especially attractive because of its high specificity, which minimizes unintended effects on non-target organisms and improves the safety profile of RNAi products. This paper describes how RNAi functions, its current applications in agriculture, the current regulatory views of RNAi-based pesticides, and concludes with a discussion of current challenges for the commercial application of RNAi in agriculture. The content presented is intended to serve as a resource for regulatory agencies, policy and lawmakers, private and public institutions, and the general public to inform regulatory assessments and consumer choice decisions.

Introduction

RNAi Discovery

The Food and Agriculture Organization (FAO) estimates that up to 40% of the annual global crop production is lost to pests and pathogens (FAO

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2019). Producers have historically relied on conventional pesticides to minimize these losses. However, with increasing incidences of pest resistance and mounting concerns over the potentially harmful effects of conventional broad-spectrum pesticides (Hawkins et al. 2019; Sharma et al. 2020), new environmentally benign methods to control pests and pathogens are needed. Over the past two decades, a new technology has attracted considerable attention as a new technology for controlling plant pests and pathogens with species-specificity.

All living cells follow the central dogma of molecular biology, in which instructions to synthesize proteins are encoded in the DNA strands. DNA sequences are transcribed to single-stranded messenger RNA (mRNA) molecules, which are then translated to form proteins that generate a specific *phenotype*[1] (i.e., observable trait or biological function conferred by the protein) (Crick 1970) (Figure 1A). The RNAi mechanism acts upon the central dogma of biology to interfere with the expression of proteins, creating alternate *phenotypes*. Double-stranded RNA (dsRNA), a duplexed nucleic acid similar to single-stranded mRNA, initially triggers the RNAi mechanism; thus, exposure to dsRNA can lead to altered *phenotypes* in many organisms.

RNAi was first described as co-suppression in petunias (Napoli et al. 1990) and later termed *gene-silencing* in the worm *Caenorhabditis elegans* (Fire et al. 1998). Artificially designed and synthesized dsRNA ingested by worms reduced the expression of genes (i.e., decreased quantities of mRNAs and, therefore, of the corresponding protein) with sequences complementary to the dsRNA sequence (Figure 1B). The reduced gene expression (i.e., silencing) led to a *phenotype* resembling mutated worms that lack the gene altogether (Fire et al. 1998). Since this seminal work in *C. elegans*, RNAi has been described in most plants, fungi, and animals (Boutros et al. 2008; Ipsaro et al. 2015) and is thought to be a natural form of cellular defense against foreign nucleic acids, such as viruses and *transposons*, which can disrupt normal cell function (Obbard et al. 2009; Swevers et al. 2018; Wallis et al. 2019).

The ability to selectively silence or *knockdown* a gene (i.e., reduce the production and quantity of mRNA of a targeted gene and its encoded protein by RNAi) was first used as a tool to study gene function (Hammond et al. 2001; Mocellin et al. 2004; Zimmer et al. 2019). Observing a change in *phenotype* may provide information on the role of the silenced gene. However, researchers soon recognized RNAi's potential to *knockdown* gene expression in crops to produce beneficial traits. For example, *knockdown* of a gene encoding a cell wall degrading enzyme in tomatoes created the "Flavr Savr™" tomato (Redenbaugh et al. 1992), a product with a slower ripening process *phenotype*, increasing the produce shelf life. Oilseed crops have similarly been produced with increased oleic acid content using RNAi (Liu et al. 2002; Yin et al. 2007; Shi et al. 2015). Furthermore, resistance to various plant viruses has been demonstrated using RNAi-inducing transgenes in tomatoes (Tomato Spotted Wilt Virus), bananas (Banana Bract Mosaic Virus), rice (Rice Tungro Bacilliform Virus), and papaya (Papaya Ringspot Virus), to name a few (Tyagi et al. 2008; Shekhawat et al. 2012; Mitter et al. 2016).

Over the past decade, a growing number of researchers have explored RNAi's potential to protect crops against insect pests. Much of this research has been driven by the remarkable finding that orally delivered dsRNA can induce an RNAi response in some insect species (Baum et al. 2007; Mao et al. 2007; Singh et al. 2013; Tayler et al. 2019; Jacques et al. 2020; Joga et al. 2016; Cagliari et

[1] Italicized terms (except genus/species names and gene names) are defined in the Glossary.

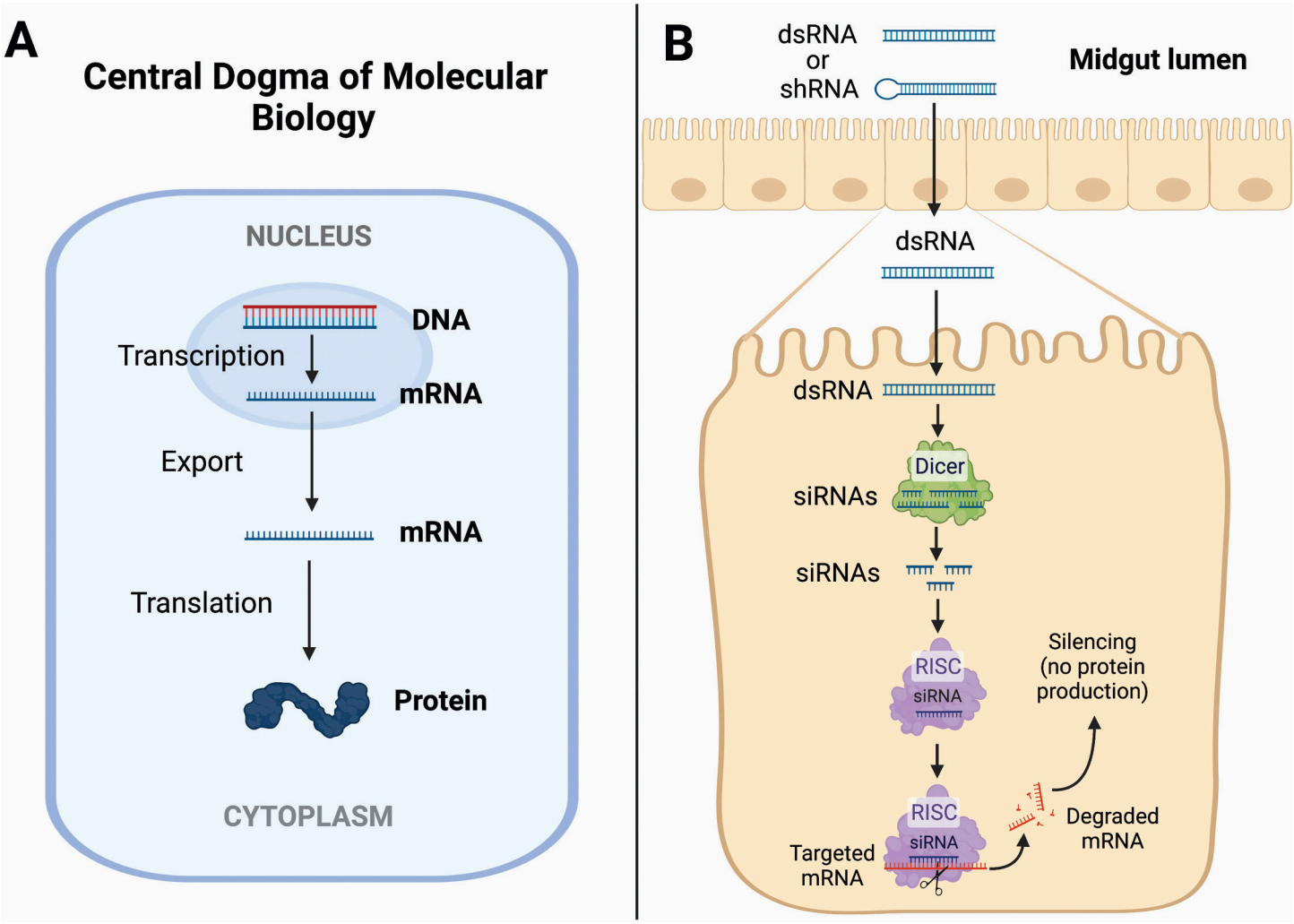

Figure 1. Mechanisms of gene silencing.
A. Central dogma of molecular biology; DNA is transcribed into mRNA, followed by translation into a protein.
B. RNA interference mechanism in insects, dsRNA matching a mRNA sequence triggers its degradation.
mRNA = messenger RNA; dsRNA = double-stranded RNA; shRNA = single hairpin RNA; siRNA = small interference RNA.

al. 2019). Targeting vital genes makes it possible to use dsRNA as a bioinsecticide (Liu et al. 2020). Most insecticidal RNAi applications focus on the targeted knockdown of genes essential for cellular functions, insect growth, or development (Kola et al. 2015). Since RNAi is highly dependent on sequence complementarity (i.e., sequence match between the dsRNA and the mRNA of the insect), dsRNAs can be designed to selectively control pest species while avoiding effects on non-target species (Baum et al. 2007; Whyard et al. 2009; Vogel et al. 2019). However, not all economically important insect pests are equally susceptible to ingested dsRNAs because of differences in dsRNA processing and physiological barriers such as gut enzymes that degrade dsRNA or dsRNA sequestration inside cells (Shukla et al. 2016; Singh et al. 2017). Hence, much of today's research on dsRNA bioinsecticide development focuses on understanding the biological factors contributing to a low or lack of an RNAi response after oral exposure to dsRNA and developing formulations to improve dsRNA efficacy.

Newly developed RNAi-based management technologies have similarly targeted fungal pathogens. However, like insects, not all fungi have conserved RNAi pathways, and sensitivity to dsRNA gene suppression varies among fungal species (Billmyre et al. 2013). The application of RNAi technology to control fungal plant pathogens also depends on overcoming the cell wall's structural and physiological barriers and targeting the actively growing part of fungal hyphae that can take up dsRNA (Šečić et al. 2021).

This review examines successful applications of RNAi-based technologies to improve crop plant yields and provide resistance to crop pests and pathogens. We will also discuss which RNAi applications have already been commercialized and some of the benefits and challenges associated with new RNAi applications in agriculture.

Use of RNAi for Crop Protection

RNAi for insect and fungal plant pathogen management works by designing a dsRNA with sequence complementary to a targeted mRNA encoding a vital protein for the insect or pathogen to survive or reproduce. After pesticide exposure and the dsRNA molecule enters a cell, the enzyme Dicer cleaves it into short interfering RNAs (siRNAs). These siRNAs are incorporated into an assembly of proteins called the RNA-induced silencing complex (RISC) and serve as guides in the cell to find matching (complementary) mRNA sequences. Once identified, a protein in the RISC degrades the complementary mRNA inherent to the pest (Figure 1B). Consequently, the protein levels encoded by the targeted mRNA are suppressed, resulting in *gene silencing* or *knockdown* (Okamura et al. 2004). The function of the targeted gene carried out by its protein is reduced or eliminated. Targeting genes necessary for the insect or pathogen to function can lead to mortality or harm to development and reproduction while not affecting other organisms. Since a complementary match between dsRNA and the targeted organism's mRNA must exist for an RNAi response, the lack of sequence match to a non-targeted organism's mRNA limits the negative impact of insecticidal dsRNA.

RNAi offers a unique and specific tool as an alternative to synthetic-chemical pesticides. An increase in the availability of *genome* databases for different species has made it possible to design species selective and efficient dsRNA molecules with negligible off-target effects within the species (i.e., knockdown of unintended genes) or impact on other organisms. These features present environmental advantages over chemical pesticides, which are generally broadly active against targeted and non-target species. Additionally, dsRNA has low persistence and is rapidly inactivated in the environment (Bachman et al. 2020; Dubelman et al. 2014; Fischer et al. 2017 and 2020; Parker et al. 2019), compared to some chemical pesticides that can persist for extended time periods.

Currently, pesticidal dsRNA molecules can be delivered to targeted organisms using two approaches: (1) by production in *genetically modified* (GM) plants or (2) by topical application (e.g., spray) of formulated products (Ivashuta et al. 2015) (Figure 2). dsRNA expression in GM plants offers the advantages of constant production throughout the growing season and protection from environmental degradation processes that may reduce dsRNA effectiveness. However, plants producing pest-specific dsRNAs are obtained through technology under GM regulations, which undergo a lengthy registration process. Sprayable dsRNA applications have potential time and cost advantages over GM crops. Producing topical dsRNA by biochemical reactions or fermentation processes is rapid and scalable compared to breeding an RNAi trait within a crop of interest. Another advantage is that plants treated with sprayable RNAi are deemed non-transgenic (non-GM). Therefore, sprayable

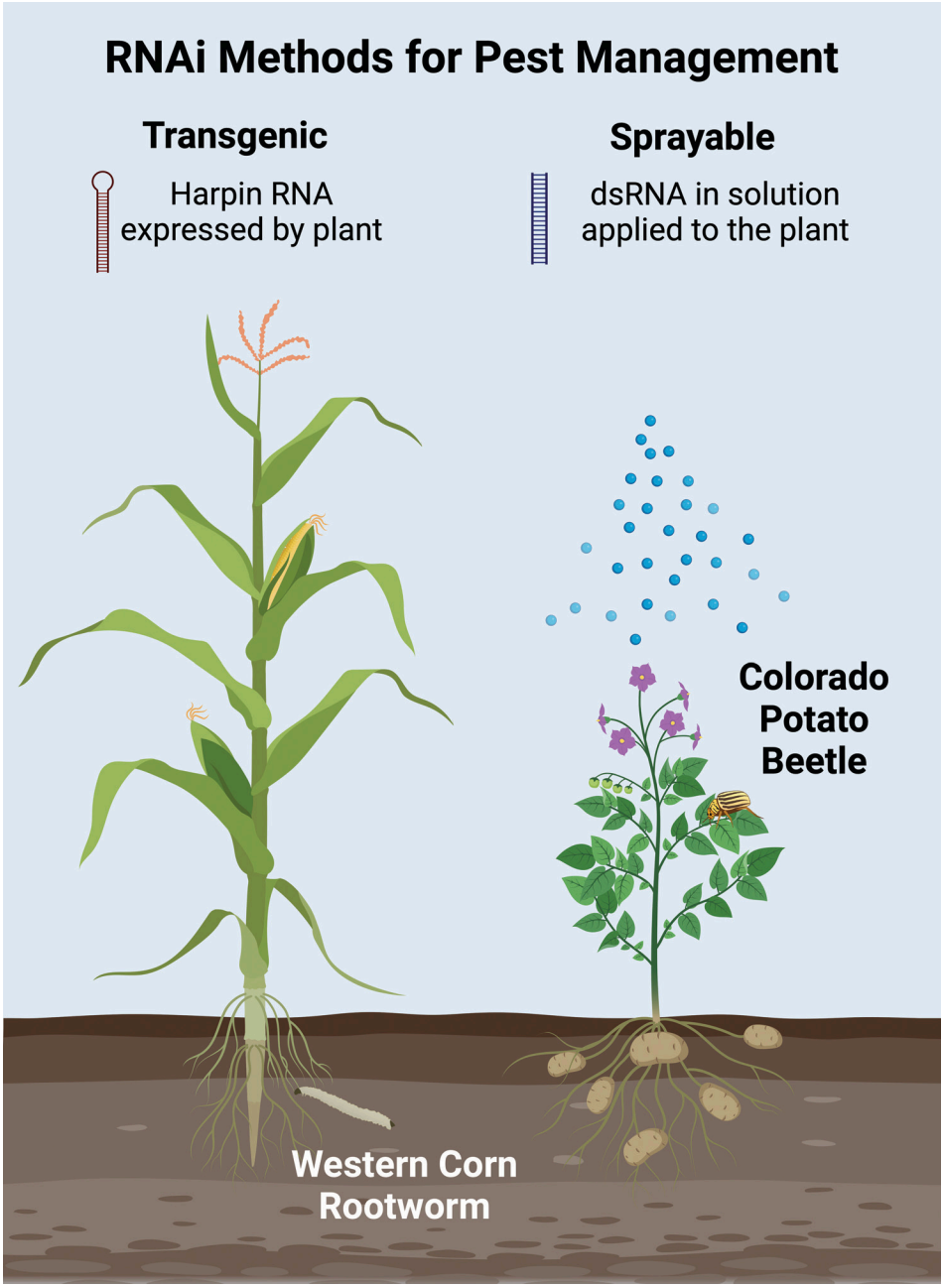

Figure 2. Approaches to deliver dsRNA used to manage insect pests: via *genetically modified* or *transgenic* plants or sprays.

dsRNA can be considered biochemical or biochemical-like product and the timeline to registration will depend on the final product composition and use (Dietz-Pfeilstetter et al. 2021; Galli et al. 2024).

Agricultural RNAi-based products are still relatively new, with only a few currently available or in late-stage development for open-field use (Head et al. 2017; Rodrigues et al. 2021a; 2021b). Many other promising RNAi-based products are in earlier stages of development. Some specifics of commercially available products and those in development are provided below.

Genetically Modified Plants Expressing dsRNA Targeting Insects

Early commercial efforts to control insects with RNAi focused on delivering insecticidal dsRNAs through *genetically modified* or *transgenic* plants, as it was not considered economically feasible to produce synthetic dsRNAs in sufficient quantities for foliar sprays. One of the first successful examples was corn expressing dsRNA targeting an essential gene, *vATPaseA*, in the western corn rootworm (*Diabrotica virgifera virgifera)*. GM plants produced sufficient dsRNA to either stunt or kill root-feeding larvae, preventing significant damage to the roots and protecting crop yield (Baum et al. 2007). In the same year, another research group demonstrated the protection of cotton plants from cotton bollworms by expressing a dsRNA targeting a vital detoxification enzyme, thereby rendering the insect susceptible to natural defense compounds present in cotton (Mao et al. 2007). In 2017, a GM corn expressing the Bt Cry3Bb1 and Cry34/35 insecticidal proteins and a dsRNA targeting the *DvSnf7* gene, a component of a complex required for cellular vesicle transport in corn rootworm, became the first GM plant to be registered by the US Environmental Protection Agency (EPA) with an insecticidal dsRNA trait. The product was commercialized as SmartStax® PRO (Head et al. 2017) in 2022. Other experimental crops have similarly been engineered to contain transgenes that express dsRNA, including tobacco resistant to fall armyworm (Xiong et al. 2013) and aphids (Mao and Zeng 2014), potatoes resistant to Colorado potato beetle (Guo et al. 2018), and cotton that negatively impacted reproduction of spider mites (Shen et al. 2017). However, these are proof-of-concept examples and have not progressed into commercialization.

Most RNAi-based GM plants express dsRNA in the nucleus. Yet, expression in the chloroplast is an attractive alternative for insect management because it avoids processing the dsRNA into siRNAs by nucleases in the plant cytoplasm. Production of intact dsRNA is desirable in cases where dsRNA instead of siRNAs is more effective in initiating the RNAi response, as is the case for many insects (Bolognesi et al. 2012; Zhang et al. 2015a; Bally et al. 2016; Bally et al. 2018; Jin et al. 2015). Cytoplasmic accumulation of dsRNA is sufficient to control some herbivorous insects (e.g., corn rootworm), but for others (e.g., Lepidoptera), chloroplast dsRNA expression may provide a means to deliver a lethal dsRNA dose.

Genetically Modified Plants Expressing dsRNA Targeting Microbial Pathogens

The demonstration that microbial pathogens are inhibited by dsRNA targeting their essential genes raised the possibility that RNAi technology could protect plants (Koch and Kogel 2014; Cai et al. 2018a; Rosa et al. 2018; Liu et al. 2020). This strategy was inspired by the discovery of natural "cross-kingdom RNAi" (ckRNAi), whereby host and microbes co-evolve bidirectional exchange of RNAs that regulate gene expression by RNAi (Weiberg et al. 2013; Buck et al. 2014; Zhang et al. 2016; Chakravarty and Massé 2019). A still unknown number of microbial pathogens use ckRNAi mechanisms to suppress host plant cell immunity and thus increase their virulence. Conversely, plant hosts also transfer small RNAs (sRNAs), including siRNA and miRNA, into pathogens and pests to inhibit their virulence (Rutter and Innes 2017; Cai et al. 2018b). For example, sRNA-containing vesicles accumulate at the infection sites and are taken up by the fungal cells and induce the silencing of fungal genes critical for pathogenicity (Zhang et al. 2016; Nasfi and Kogel 2022; Ruf et al. 2022).

The *gene silencing* efficacy of dsRNAs in a colonizing microbe has been experimentally demonstrated in several cases in which the dsRNA is produced in a GM plant. The plant cellular silencing machinery subsequently dices this dsRNA to siRNA duplexes that are eventually transferred to the microbe and guide the silencing of target genes. Such engineered RNA-based communication, termed host-induced gene silencing (HIGS) (Nowara et al. 2010), has emerged as a promising strategy for pathogen crop protection. A wide range of experimental GM crops expressing dsRNAs targeting essential and/or pathogenicity genes of viruses, viroids, bacteria, fungi, oomycetes, and nematodes have been reported (Nunes and Dean 2012; Koch and Kogel 2014; Qi et al. 2019), but to date they are only a proof of concept.

Sprayable dsRNA for Spray-induced Gene Silencing (SIGS)

The discovery of RNAi for pest management stimulated research to develop technology for sprayable dsRNA products for field-scale application. The economic feasibility of sprayable dsRNA for plant health was initially hindered by the high cost of producing large quantities of material needed for broad acreage use. However, this problem was overcome by developing efficient methods of dsRNA biosynthesis. Cell-free production can deliver kilogram-scale quantities of dsRNA at a cost of less than US $1 per gram (Rodrigues et al. 2021). Several organizations are pursuing microbial production of dsRNA (see, for example, Hashiro et al. 2021). Continual innovation for even more efficient dsRNA production and delivery will drive down the cost of goods, potentially opening new market opportunities for RNAi-based pest control. EPA registration for the first sprayable dsRNA biopesticide was approved in December 2023 (US EPA 2023b). The active ingredient, named ledprona, targets the Colorado potato beetle protein that functions to degrade damaged proteins (Rodrigues et al. 2021).

Effective uptake of dsRNA by target organisms or crop plants is critical to

the success of sprayable RNAi. Some agricultural applications, for example, plant viral disease resistance, rely on dsRNA internalization and systemic spread within the target crop (Mitter et al. 2017a; b). In this case, dsRNA must cross the plant cuticle and cell wall to initiate amplification and systemic spread of anti-viral siRNA. Insect control by RNAi depends on contact and uptake of intact dsRNA by target pests (Christiaens et al. 2020). In general, insects in the order Coleoptera (beetles) are susceptible to feeding on a dsRNA as a trigger for insecticidal RNAi (Fishilevich et al. 2016; Baum et al. 2007; Zhu et al. 2011; Petek et al. 2020; San Miguel and Scott 2016). These insects efficiently process orally ingested dsRNA to initiate a strong RNAi response, resulting in insect mortality and decreasing plant damage. In contrast, insects in orders Lepidoptera (caterpillars) (Terenius et al. 2011; Kolliopoulou et al. 2014; Lim et al. 2016) and Hemiptera (true bugs) (Christiaens and Smagghe 2014; Jain et al. 2020) are less susceptible to ingested dsRNA. However, many insects with a reduced RNAi response from dsRNA feeding mount an effective RNAi response when the dsRNA is injected, supporting the idea that the cellular RNAi machinery is less accessible to orally delivered dsRNA. To trigger RNAi, a sprayable applied dsRNA to a plant must be ingested and survive the insect midgut environment until internalization into midgut cells for processing into siRNA effector molecules. Gut pH, digestive enzymes that degrade dsRNA, dsRNA entrapment within intracellular vesicles, lack of uptake, and lack of cell-to-cell spread of the RNAi signal throughout the organism are all factors that can limit the RNAi response to orally delivered dsRNA in insects (Christiaens et al. 2020).

Methods of dsRNA formulation with specialized delivery agents are being developed to overcome biological barriers to sprayable RNAi (Zhu and Palli 2020). Susceptible organisms such as the Colorado potato beetle can be controlled by simple formulations of naked dsRNA (Zhu et al. 2011; Maximo et al. 2020; Mehlhorn et al. 2020; Petek et al. 2020). On the other hand, insect pests with a limited response to oral RNAi require delivery agents to protect against digestive enzymes, facilitate cell uptake, and promote release within insect cells (Christiaens et al. 2018b; Christiaens et al. 2020). Virus control requires delivery across plant surfaces to reach plant cells and phloem (Mitter et al. 2017a; b). Delivery agents in development include simple chemical compounds like ethylenediaminetetraacetic acid (EDTA) and more highly engineered *nanoparticles*, including liposomes, cationic polymers, basic peptides, and clay *nanosheets* (Whyard et al. 2009; Das et al. 2015; Mitter et al. 2017b; Christiaens et al. 2018b; Christiaens et al. 2020; Zhu and Palli 2020). Microorganisms expressing dsRNA are also used as another route of delivery (Goodfellow et al. 2019); example organisms engineered for dsRNA expression include the *Escherichia coli* bacterium (Tian et al. 2009; Zhu et al. 2011), the yeast *Saccharomyces cerevisiae* (Murphy et al. 2016; Zhong et al. 2019; Duman-Scheel 2019), and insect bacterial *symbionts* (Whitten et al. 2016; Whitten and Dyson 2017; Andongma et al. 2020).

Direct application of dsRNA to the body surface of insects has shown mixed results. In insects with particularly rigid exoskeletons, such as beetles, there are no reports of successful RNAi induction by physical contact. As the exoskeleton of insects is covered with a waxy epicuticle impermeable to water and water-soluble molecules, the difficulty of dsRNA to bypass the exoskeleton is not surprising (Gibbs 1998). However, several cases of gene *knockdown* by sprayable dsRNAs have been reported. Sprays of dsRNA onto first instar larvae of the Asian corn borer and the cotton bollworm caused significant mortality in these insects (Zhang et al. 2015b). Sprayable dsRNAs also induced effective *knockdown* of targeted *transcripts* in pea aphids (Niu et al. 2019). In these cases, the mode of dsRNA entry was not identified, but it was suspected that the dsRNA could penetrate the thinner flexible portions of the cuticle between joints or *spiracles* (insect openings used for gas exchange), allowing passage into the *hemolymph* (insect equivalent to human blood). With only a few reports of sprayable dsRNAs penetrating the exterior of insects, sprays will likely be limited to some soft-bodied species or to those cases where environmentally safe cuticular penetrants can be found.

Methods for field application of dsRNA include spraying (spray-induced gene silencing or SIGS), soil drenches, seed coats, baits, and plant injection (Cagliari et al. 2019). Spraying dsRNA is the simplest application method for the control of crop pests. Water soluble or emulsified formulations of dsRNA are used for spraying crops or fruits, similar to the application of traditional crop protection agents. High-pressure sprays have also been shown to deliver siRNA into plants for *gene silencing* (Dalakouras et al. 2016). The success of the SIGS approach in the field is highly dependent on tailoring the dsRNA and formulation to the target organism. Other crop applications may require more sophisticated formulation to achieve commercially acceptable levels of efficacy. Products targeting plant viruses benefit from *nanocarrier* particles that promote the uptake of dsRNA by plant tissues (Mitter et al. 2017a; b). Several pathogenic fungi such as *Botrytis cinerea* (Wang et al. 2016)*, Sclerotinia sclerotiorum* (McLoughlin et al. 2018)*,* and *Fusarium graminearum* (Koch et al. 2016) are being targeted for control by dsRNA sprays (for review see Šečić and Kogel 2021).

Baits using dsRNA as the pesticidal agent are envisioned in "attract and kill" applications in an attempt to control an array of pests, including Western corn rootworm (Schumann et al. 2014; Rodrigues et al. 2016), aphids (Jacques et al. 2020) or fruit flies (Taning et al. 2016), as a few examples. Soil and root drenches have also been demonstrated to deliver dsRNA to citrus trees and grapevines to target phloem-feeding organisms such as psyllids and other hemipterans (Hunter et al. 2012; Li et al. 2015; Ghosh et al. 2017). Trunk injections were also used to deliver RNA to woody plants, such as apples and grapes, with dsRNA persisting for at least three days (Dalakouras et al. 2018). Applying dsRNA as a seed coating is also being pursued (Renouard et al. 2014).

Sprayable dsRNA for Beneficial Insect Protection

RNAi can also be used as a viricidal

prophylactic treatment for beneficial insects such as honey bees. A dsRNA-based product concept was designed to protect against the Israeli Acute Paralysis Virus (IAPV) when fed to honey bees (Hunter et al. 2010). Similar results were obtained when bees were fed dsRNA targeting the deformed wing virus (Desai et al. 2012). Feeding specific dsRNAs to honeybees also reduced internal microsporidia (Paldi et al. 2010) and external Varroa mite (Garbian et al. 2012; Maori et al. 2019) parasite infections. In the latter case, it was reported that adult bees transmitted the long dsRNA to the mites, providing sufficient dosage to reduce the number of parasitizing phorectic mites.

Sprayable dsRNA Targeting Microbial Pathogens

In 2016, two publications showed that dsRNAs from plant tissue taken up by the fungal pathogens *B. cinerea* and *F. graminearum* induced silencing of fungal target genes, resulting in reduced virulence and less infection of the inoculated plant tissues (Koch et al. 2016; Wang et al. 2016). The possible application of sprays for control of fungal diseases is illustrated by growth inhibition of various agronomically important mycotoxin-producing *Fusarium* species that cause diseases by treatment with dsRNAs and siRNAs (Koch et al. 2016; Koch et al. 2019). Importantly, the use of dsRNA is anticipated to delay widespread resistance to current fungicides, as RNAi represents a new mode of action and may not be affected by known resistance mechanisms. Examples of the efficacy of sprayable RNAi include a foliar spray containing dsRNA targeting three detoxification enzyme genes of *F. graminearum*, which strongly inhibited fungal growth on barley leaves (Koch et al. 2016; Koch et al. 2018). Consistent with the knowledge that RNAs are mobile in the plant vasculature (Molnar et al. 2010; Melnyk et al. 2011), compromised fungal growth was observed in directly sprayed (local) as well as non-sprayed (distal) parts of detached leaves. In line with these findings, sprayable application of dsRNAs that target *BcDCL1* and *BcDCL2* of *Botrytis cinerea* reduced the virulence of the fungus (Wang et al. 2016). Sprays of dsRNA can also inhibit the growth of *B. cinerea* and *Sclerotinia sclerotiorum* on canola (McLoughlin et al. 2018). The above findings support the conclusion that fungal cells can take up environmental dsRNAs and siRNAs directly or indirectly via the plant cells. In contrast, dsRNAs expressed in GM plants are mainly processed by the plant RNAi machinery, and the siRNAs are transferred to impact pathogen gene expression and virulence.

It remains unclear what determines dsRNA uptake in fungi and whether this uptake is a common phenomenon across the fungal kingdom. Establishing the effectiveness of dsRNA uptake across a wide range of fungal microbes with different lifestyles and colonization strategies is a critical next step in developing this technology. While reproducible effects have been shown, the efficacy of sprayable naked dsRNA is probably hindered by the inability to effectively reach the inner leaves in sufficient quantity to silence the target genes of a colonizing fungus (Liu et al. 2021). Therefore, dsRNA application strategies improved by physical or chemical means may increase the efficacy of dsRNA sprayables (e.g., carbon dots (Schwartz et al. 2020)). It should also be noted that RNAi-based control strategies may not be suitable for some fungal pathogens even though they possess the RNAi machinery. A recent study of *Zymoseptoria tritici* failed to detect either dsRNA uptake or host-induced *gene silencing* of the targeted genes (Kettles et al. 2019). Furthermore, no natural cross-kingdom communication was observed between this pathogen and wheat (Ma et al. 2020). These observations highlight the importance of both uptake and expression of necessary RNAi machinery components for plant protection.

Similar dsRNA instability and delivery issues have been reported in dsRNA-mediated protection against viral diseases. The use of dsRNA loaded on non-toxic and degradable clay *nanosheets* on tobacco leaves resulted in sustained release and sustained control of pepper mild mottle (PMMoV) and cucumber mosaic (CMV) virus 20 days after application (Mitter et al. 2017a; b). Chemical formulations may not only enhance the uptake of dsRNA from leaves but may also improve penetration into fungi and persistence where needed.

Genetically Modified Crops Expressing dsRNA for Enhanced Quality Traits

Plants produce many secondary metabolites of great economic value (i.e., drugs, flavors, fragrances, pesticides, and dyes). Some plants also produce secondary metabolites for protection from insects and diseases, such as glycoalkaloids in potatoes and gossypol in cotton. With RNAi, plant genes can be silenced, enabling a change in metabolic levels at a desired step in the biosynthetic pathway, thus resulting in desirable plant properties.

Using GM RNAi plant traits have several advantages over classical mutagenesis or gene editing by CRISPR-CAS. For example, the down-regulation of multiple members of a gene family is achievable with a single dsRNA construct (Rommens et al. 2008). In cotton, silencing multiple members of a d-cadinene synthase gene produced Ultra-Low Gossypol Cottonseed (ULGCS) (Sunilkumar et al. 2006; Rathore et al. 2020). This trait, which renders cottonseed safe for human consumption, has been deregulated in the United States by the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA), and if widely adopted in cotton-growing countries, could provide ten million tons of protein, improving nutrition security (Rathore et al. 2020).

Regulatory Considerations

Product Characteristics as a Focus of Governance

As previously explained, there are two main categories of dsRNA products based on the mode of delivery: GM plants and sprayable products (Figure 2). Depending on the country, different regulatory schemes may apply to the different types of dsRNA-based products (Dietz-Pfeilstetter et al. 2021). In countries with

specific laws regulating GM organisms (GMOs), dsRNA products produced within a transgenic plant may be regulated differently than sprayable dsRNA products, which may not be regulated under a GM law unless the sprayable product is produced by or applied as a GM microbe. In some instances, the engineered microbe may be regulated under a GM law, while a dsRNA spray product may be regulated more similarly to conventional pesticides. Under a product-based regulatory scheme, such as in the United States, the individual product characteristics and the regulatory laws that apply to a product provide the basis for regulation and the types of information requested by regulatory bodies. Moreover, guidance documents provided by individual regulators (e.g., EPA Ecological Effects series 885 guidelines), scientific advisory boards (e.g., Scientific Advisory Panel for the US EPA), and consensus documents established by the Organization for Economic Cooperation and Development (OECD) provide baseline information about the relevant pathways and exposures that are useful for assessing risks of RNAi based products to human health and the environment (OECD 2020; OECD 2023).

When considering new pest management products, most regulatory bodies have a standard suite of data requirements that must be submitted. For instance, although the data needed by EPA for dsRNA products is determined on a case-by-case basis, it has published pesticide data requirements in the Code of Federal Regulations (CFR) for conventional, antimicrobial, biochemical, and microbial pesticides. Of these requirements, the EPA recommends developers use the biochemical and microbial pesticide data requirements as a guide and starting point to consider the basic types of information the EPA would need in a regulatory package to support a sprayable dsRNA product (Dietz-Pfeilstestter et al. 2021). Across regulatory bodies, the standard data requirements for pesticides/biopesticides are often similar. Still, each regulatory body will likely have some requirements specific to its relevant laws and regulations. Moreover, when considering novel products such as dsRNA pesticides, a regulatory body may require, on a case-by-case basis, additional or different data for review.

Problem formulation is a common approach used by the regulated and regulatory communities to consider policy goals, scope, assessment endpoints, and methodology to determine relevant exposure scenarios and possible consequences of those exposure scenarios for risk assessment (Wolt et al. 2010). Performing a problem formulation analysis allows risk assessors to determine the specific characteristics of a product that are important to the risk assessment (Wolt et al. 2010; Romeis and Widmer 2020; De Schutter et al. 2022) to ensure a product meets regulatory standards for approval. Although the discussion of the problem formulation approach herein is specific to products that work through an RNAi mechanism, this approach is often generically used by risk assessors for other types of products (e.g., GM plants and plant-incorporated protectants). Thus, problem formulation is a good approach to determining what additional data might be needed for a regulatory decision. In this approach, an initial step is to understand what the product is (e.g., sprayable dsRNA or GM plants expressing dsRNA product) and its characteristics (e.g., does the formulation stabilize the dsRNA in the environment, facilitate uptake of the dsRNA by the target organism(s), are there contaminants or impurities), routes of exposure for humans and non-target organisms by understanding how and where the product will be applied (e.g., in planta or ground or aerial application methods, application rate, specific crops), and the purpose of the product (e.g., increase disease resistance, control insect pests). In addition, bioinformatic comparisons of the dsRNA with gene sequences of relevant non-target species and results of preliminary empirical testing for effects on non-target species are helpful during the problem formulation stage to inform the breadth of non-target organisms that may need evaluation in a risk assessment to determine the range of activity for a dsRNA product. Similarly, in the early stages of product development, bioinformatics comparison of the dsRNA sequence with human gene sequences informs the human health risk. Although information on the mode of action is useful to inform the risk assessment, a complete understanding of the mode of action is not necessary to conduct a risk assessment. The answers to these initial questions form the basis for determining the most relevant pathways of exposure and potential hazards for target and non-target organisms and humans, for a specific dsRNA product and what, if any, of the standard suite of data elements would be needed to make a regulatory decision.

An important resource in the problem formulation process for pesticides with novel characteristics is the inclusion of scientific advisory boards. For instance, under the Federal, Insecticide, Fungicide, and Rodenticide Act (FIFRA), the EPA can convene Scientific Advisory Panels consisting of external scientific experts on a topic such as RNAi. These expert panels are important and can advise on relevant studies, information types, and the most current available science. For example, input from two EPA-convened scientific advisory panels was helpful to the EPA in its review of the novel *DvSnf7* dsRNA product to control corn rootworm (FIFRA SAP 2014; FIFRA SAP 2016).

Current Regulatory Landscape

Products for which dsRNA is Produced Within the Plant

For GM plants producing dsRNA, the regulatory structure of each country will determine the regulatory body that will assess a regulatory application. In the United States, the Coordinated Framework for Regulation of Biotechnology, drafted in 1986 and updated in 2017 (OSTP 1986, White House 2017), describes the roles and responsibilities of the EPA, USDA, and FDA concerning *biotechnology* oversight. For *transgenic* plants producing dsRNA, the Coordinated Framework applies to GM plant with an RNAi mode of action. The EPA's role in the Coordinated Framework for dsRNA producing GM plants with a pesticidal trait is to regulate them under FIFRA as plant-incorporated protectants. EPA regulates the sale, distribution, and use of all pesticides including those produced through genetic engineering and evaluates risks to humans and the environment from exposure to pesticides, including dietary exposure to pesticide residues

in human and animal food. Under the Federal Food Drug and Cosmetic Act (FFDCA), the EPA also sets tolerances (i.e., maximum residue limits or MRLs) and exemptions from the requirement of a tolerance for residues of pesticide products in food and feed commodities, which the FDA enforces. Here it is relevant to note that residues of a pesticidal dsRNA plant-incorporated protectant are exempted from the requirements of a tolerance as part of the existing nucleic acid exemption for plant-incorporated protectants (40 CFR 174.507). The FDA's regulatory role relating to dsRNA producing GM is to offer a voluntary food safety consultation process to help ensure the resulting food and/or feed is safe for human/animal consumption. The USDA regulates GM plants with an RNAi trait under the Plant Protection Act if it determines that the plant poses a plant health risk compared to conventional plants. In the United States, a single RNAi plant product may be regulated by up to three Agencies depending on the specifics of the product. In Canada, dsRNA products produced within the plant are regulated by the Canadian Food Inspection Agency, and if the trait is in a food plant, Health Canada (CFIA 2019). In countries with laws specific to GM organisms, dsRNA traits produced within the plant would be regulated under those laws and the bodies responsible for regulation.

Several regulatory bodies worldwide have knowingly or unknowingly (e.g., virus-resistant papaya — at the time of deregulation, the underlying mechanism for the trait was unknown) approved *transgenic* plants containing dsRNA traits. In the United States, the only currently registered pesticidal RNAi products are plant-incorporated protectants that are produced within the plant (e.g., C5 honeysweet plum to control plum pox virus, MON87411 corn containing the *DvSnf7* dsRNA for corn rootworm control [US EPA 2010a; US EPA 2015a]). In addition to pesticidal products, other *transgenic* RNAi products on the market may silence genes to improve agronomic traits or consumer desirability of produce (e.g., non-browning Arctic® apple [Waltz 2015]; high oleic soybean [ISAAA 2023a]; low lignin alfalfa [ISAAA 2023b]). The ULGCS trait in cotton required approval only from USDA-APHIS and FDA and not from EPA as the trait did not result in the production of any new chemical in the plant but rather the dsRNA was limited to the suppression of a naturally occurring terpenoid in the seed (Rathore et al. 2020). Several other GM RNAi products, such as tomatoes resistant to tomato spotted wilt virus, are also in development (USDA APHIS 2019). In the European Union (EU), the European Food Safety Authority (EFSA) completed assessments for potato, soybean, and MON87411 corn crops containing dsRNA traits (Mezzetti et al. 2020). Canada has also approved MON87411 corn for corn rootworm control, high oleic acid soybean and low lignin alfalfa (Health Canada 2017; ISAAA 2023b).

Sprayable dsRNA Products

In the United States, Canada, and the EU, sprayable dsRNA products (except for some microbial products as indicated under the product characteristics section) that act as a pesticide by *post-transcriptional gene silencing* are regulated like a conventional pesticide since the dsRNA is not a living organism, nor does it impart a heritable trait. In the United States, the EPA regulates sprayable dsRNA products intended for pest control as pesticides under FIFRA and sets tolerances or exemptions from the requirement of a tolerance for residues of the dsRNA in food or feed under the FFDCA if the product is intended to be used on food crops. The FDA regulates sprayable products that are classified as animal drugs. Only one foliar dsRNA product is registered with the EPA in the United States for its use in potatoes to control the Colorado potato beetle (Rodrigues et al. 2021a, 2021b; US EPA 2023b). The Pest Management Regulatory Agency regulates sprayable dsRNA products for pesticide use in Canada. Although sprayable pesticide formulations have not yet been registered, sprayable RNAi products are being developed (Yan et al. 2020).

Ecological Risk Assessments

Considerations for ecological risks associated with RNAi products that need to be addressed in a risk assessment during the regulatory approval process include different exposure pathways, environmental fate of the dsRNA product, and potential effects on non-target organisms. The product type (i.e., sprayable or produced within the plant) will determine which exposure pathways and non-target organisms are relevant.

Potential Exposure Pathways

Products Containing a dsRNA Trait in the Plant

Of the relevant pathways to exposure for non-target organisms, the most likely exposure pathway related to dsRNA traits expressed within the plant is through oral consumption of the plant or plant materials (e.g., pollen). Other exposure routes to non-target organisms are possible but unlikely, despite the likelihood that dsRNAs will be expressed at relatively high levels in plant tissues because the dsRNAs and siRNAs are likely to be rapidly degraded as plant tissue decomposes (US EPA 2015b).

Sprayable dsRNA Products

Similar to dsRNA produced within the plant, the most common exposure pathway for sprayable dsRNA formulations is likely to be oral (dietary) exposure, where the product is applied to the targeted location, and the target or non-target organism consumes material sprayed with the dsRNA (OECD 2020). However, in addition to dietary exposure, sprayable dsRNA formulations could result in exposure to the dsRNA product through topical and aquatic exposure routes, similar to conventional pesticides (OECD 2020). However, the sprayable dsRNA products that would cause *post-transcriptional gene silencing* through these other exposure routes would need to be formulated to penetrate physical barriers such as a plant membrane or insect cuticle. Product formulation may impact both uptake and the ability to penetrate these physical barriers at high enough levels to achieve an effect on non-target organisms through sprayable exposure. Adjuvants, detergents, *nanocarriers*, or other agents that facilitate RNA movement across membrane barriers (Castellanos et al., 2019; Christiaens et al. 2020; OECD 2020; Martinez et al. 2021) could impact dsRNA uptake by non-target organisms. Additive agents could also improve the

stability of dsRNA in the environment, resulting in dsRNA being environmentally available to ensure greater exposure to target organisms for longer periods of time before being degraded; in such cases, the potential for increased exposure to non-target organisms also needs to be considered.

Hazard Characterization Effects on Non-Target Organisms

A key to understanding the possible effects of dsRNA on non-target organisms is to understand how different organisms respond to environmentally available RNAs. The RNAi machinery is typically only found in eukaryotic organisms, such as fungi and insects, and not in prokaryotes, like bacteria or archaea (OECD 2020). Importantly, even in organisms in which the RNAi machinery is found, there exists a high degree of variability in the efficiency of an RNAi effect across taxonomic groups (Christiaens et al. 2018a; b). For instance, when considering the susceptibility of non-target insects to RNAi, Coleoptera (beetles) tend to be highly sensitive. In contrast, other groups of insects, including Hemiptera (aphids, cicadas, and plant bugs), Orthoptera (crickets and grasshoppers), Hymenoptera (bees, wasps, and ants), and Lepidoptera (moths and butterflies) display more significant variability in their responses (Christiaens et al. 2018b; Christiaens et al. 2020). This differential sensitivity may influence study design depending on the sensitivity of the specific test species, specific life stages of a species that may be affected, study duration, which may need to be longer than normal, and *sublethal endpoints*, which might need to be evaluated to provide adequate information for environmental risk assessors (OECD 2020). Sensitivity is also important when considering the amount of dsRNA non-target organisms are likely to encounter in the environment. For GM plants, typical tests that would be submitted to the EPA are performed under a maximum hazard dose paradigm in which the test dose is typically at least ten times higher than the highest expected concentration of the active ingredient (e.g., the dsRNA) in the field, which provides a conservative estimate that can account for all possible exposures that might occur in the field (US EPA 2010b). The expected environmental concentration for a transgenic1 plant dsRNA product would be derived from the amount of dsRNA within plant tissues at a particular plant life stage. In its review of the *DvSnf7* dsRNA produced in *transgenic* corn, the EPA used the amount of dsRNA in dry leaf tissue as a conservative estimate of the highest expected environmental concentration (US EPA 2016a).

In addition to the general efficiency of the RNAi effect across taxonomic groups, other considerations for understanding the potential for impact on non-target organisms include which organisms are likely to be exposed, how closely related the non-target organisms are to the target pest, and whether any protected non-target species may be exposed. These considerations are likely to vary for individual products. For instance, a dsRNA product targeting a pest in corn may result in exposure to different non-target organisms than a product targeting a pest in a tomato crop.

To illustrate the possibilities of non-target organisms that may be exposed to a product, a helpful example is the EPA's review of *transgenic* corn producing the *DvSnf7* dsRNA to manage corn rootworm. The EPA does not have codified data requirements specific to genetically engineered products but typically requires data evaluating the potential for effects of a pesticide on a standard suite of birds, mammals, non-target insects, honeybees, plants, and aquatic animal species (US EPA 2016a). Furthermore, the nontarget insects tested are typically determined on a case-by-case basis depending on expected exposure (e.g., test species may differ for GM corn vs. cotton). When the EPA assessed the *DvSnf7* product for non-target insects, it required data for the ladybird beetle *Coleomegilla maculata*, a parasitic wasp, the insidious flower bug *Orius insidiosus*, and a predatory carabid beetle; all species commonly found in corn fields that would be potentially exposed to the *DvSnf7* dsRNA (US EPA 2016a).

There are two main ways to determine the range of activity of the pesticide on non-target organisms: an empirical approach using *bioassays* and a *bioinformatics* approach comparing the sequences of siRNAs generated from the dsRNA to the *genomes* or *transcriptomes* of non-target species (OECD 2020). The empirical *bioassay* approach tests a range of species, starting with close relatives of the targeted pest and then moving to more distantly related species. The *bioinformatics* approach can provide additional information on the potential for impacts on non-target organisms (US EPA 2016a) and help select which non-target organisms to test. However, these *bioinformatics* comparisons are not predictive of toxic effects (i.e., a match is not indicative of a hazard) and are limited by the number of species for which *genomes* have been sequenced (US EPA 2016a). Consequently, at this time, *bioinformatics* comparisons cannot be used alone to identify a risk of concern.

Considerations for sprayable products, such as species sensitivity, the likelihood of exposure, and relatedness to the target species for sprayable dsRNA, are similar to those for GM products mentioned above. However, for sprayable dsRNA products, the formulation will likely be an additional critical factor to consider. For products formulated to persist in the environment or penetrate barriers to uptake, the breadth of test species for which data are needed to inform a risk assessment may depend on whether the target gene is specific to a species or taxonomic group or more broadly conserved across taxa (OECD 2020). Previous efforts to increase the efficiency of dsRNA and improve uptake across barriers have used *nanoparticle* carriers and/or detergents to increase dsRNA persistence and uptake when sprayed on both plants and insects (Mitter et al. 2017; Vurro et al. 2019; Yan et al. 2020; De Schutter at al. 2021; De Schutter at al. 2022).The EPA regulated sprayable dsRNA as a biochemical or biochemical-like product, but recommend using the microbial data guidelines because they have longer study durations. Differences in required studies would likely be driven by differences in exposure potential.

Human Health Risk Assessment

Considerations for human health risk associated with RNAi products that

need to be addressed during the regulatory approval process include potential exposure pathways, considering existing barriers to uptake and the stability of the dsRNA in the formulation, as well as the characterization of the potential hazard, including toxicity, and similarity of the dsRNA nucleotide sequence to human gene sequences.

Human Health Considerations for Products Where dsRNA is a Genetically Engineered Crop

For products relying on dsRNA delivery by GM plants, as with environmental risk assessment, the main human exposure pathway is likely through dietary consumption. This is because the dsRNA is contained within plant cells, which essentially eliminates other routes of exposure for humans. As noted earlier, the EPA has issued an exemption from the requirement of a tolerance for residues of nucleic acids that are part of a plant-incorporated protectant (CFR 2007). In a 1992 guidance document FDA stated that "nucleic acids are present in the cells of every living organism, including every plant and animal used for food by humans or animals, and do not raise a safety concern as a component of food. In regulatory terms, such material is presumed to be generally recognized as safe."

During its review and approval of the *DvSnf7* trait for corn rootworm control, the EPA considered oral toxicity data to ensure the product's safety (US EPA 2016b). Additional information helpful for evaluating the risk of dsRNA products to human health includes *bioinformatic* studies evaluating matches and mismatches of the 21 bp sequences of potential siRNAs derived from the dsRNA with sequences of the human *transcriptome,* and a consideration of the barriers to dsRNA uptake in the human body (US EPA 2016b). When dietary dsRNA consumption is the most relevant pathway, studies evaluating potential dietary toxicity in mammals, often mice and rats, are appropriate. In granting the registration for *DvSnf7*, the EPA reviewed a comparison of endogenous corn siRNA sequences with the human *transcriptome* and noted that there were approximately 150 endogenous corn siRNAs with 100% sequence matches to human protein-coding *transcripts*. The EPA concluded that this result further supports the history of safe consumption of endogenous plant small RNAs and that humans and mammals regularly consume unmodified "naked" RNA without experiencing adverse effects (Petrick et al. 2013; Rodrigues and Petrick 2020), indicating that humans have barriers preventing the uptake of such dsRNA (US EPA 2016b).

When considering the potential for RNAi effects on humans in the context of the *bioinformatics* information noted above, risk assessors should also consider the challenges associated with creating human therapeutic treatments that can be delivered to the targeted location in the human body (Christiaens et al. 2020; OECD 2020). Achieving sufficient dsRNA uptake through oral exposure to produce an RNAi effect in humans is challenging because of low oral bioavailability and numerous barriers preventing dsRNA uptake in the human body. These barriers include *nucleases* in saliva and the gastrointestinal tract, acidic conditions in the stomach, and numerous membranes through which the dsRNA and subsequent siRNAs must pass to enter the bloodstream (US EPA 2016b). Evidence suggests that the human body rapidly clears short RNAs from circulation (OECD 2020). In overcoming these hindering factors, the administration of dsRNA for human therapeutic purposes involves formulations to protect the gastrointestinal tract from degradation and facilitate movement across membrane barriers toward the targeted tissues (OECD 2020). Consequently, because dsRNA produced by GM plants cannot easily be protected through formulation changes, barriers to uptake remain in the human body, preventing uptake through oral exposure at levels expected to cause an adverse effect.

Human Health Considerations for Sprayable dsRNA Products

For sprayable dsRNA products, in addition to dietary exposure, other exposure pathways such as dermal, ocular, and inhalation may be possible depending upon the method of appliaction, as they would be for conventional pesticides (OECD 2023). Thus, information may be needed to assess the mammalian toxicity of the dsRNA and the formulated product via oral, inhalation, ocular and dermal contact, depending on the specific product and its use patterns. Regarding the dietary risk consideration, it is relevant to note that, unlike dsRNAs in plant-incorporated protectants, the residues of new sprayable dsRNA products would be required to obtain a new exemption from a tolerance if they are proposed for use on food corps, as the existing nucleic acid exemption only applies to plant-incorporated protectants.

In addition to empirical studies assessing toxicity via these exposure routes, a bioinformatic analysis as described for GM crops and in a recent OECD publication (OECD 2023) is also critical for assessing potential risks to human health associated with sprayable dsRNA products. For sprayable products, the possibility for non-sequence specific immune responses should also be considered as part of the hazard assessment (OECD, 2023).

Additionally, the formulation and chemical modification of sprayable dsRNA products are critical factors to consider when assessing risks to human health, as they may increase the likelihood for exposure to the dsRNA (OECD, 2023). As noted above for GM crops, barriers to uptake found in the human body are likely to prevent uptake of sufficient levels of "naked" dsRNA resulting in an adverse effect. Thus, a human health risk would not be expected unless a product is formulated or the RNA is otherwise modified to penetrate a barrier(s) (i.e., through the oral, dermal, ocular, or inhalation exposure route) to uptake in humans. Therefore, factors like the stability of the dsRNA (e.g., a formulation that makes the RNA more stable and persistent) or likeliness to penetrate a barrier(s) to uptake, such as the skin, will determine the types and duration of studies and information needed for a regulatory agency to make a regulatory decision (OECD 2020). In cases where a product is formulated to increase stability, persistence, or overcome a barrier to uptake, the formulated end-use product may require submission of product-specific data for each formulated product to assess potential risks to human health.

International Coordination

International coordination by regulatory bodies results in a more streamlined and consistent global regulatory process, which benefits developers, agricultural production, and trade. When data requirements are similar across international regulatory bodies, developers are more equipped to include all necessary information for a regulatory dossier, producing a more streamlined process and potentially faster time to market. Although several benefits accrue from coordination and harmonization between international regulatory bodies, this process can be hindered by regulating products in individual jurisdictions with unique laws and regulations. To complicate coordination efforts further, laws and regulations within one jurisdiction can be complex, and in some cases, a single product may fall under the jurisdiction of multiple regulatory agencies within a single country (OSTP 1986; White House 2017). As a result, coordination between countries may involve multiple regulatory agencies, each regulating under different laws and regulations.

Despite these difficulties across and within jurisdictions, different regulatory bodies are making efforts to outline the most common considerations for dsRNA products. In the broadest effort, regulatory bodies from the United States, Canada, Germany, and EFSA worked together under the auspices of the OECD to publish documents outlining the most critical considerations for assessing the environmental risk of dsRNA products (OECD 2020; OECD 2023). These documents guide considerations that need to be addressed and provide background information to guide academic scientists, developers, and regulators in developing valuable information for risk assessment of sprayable dsRNA products.

Challenges

RNAi is an emerging pesticidal technology that will positively impact sustainable agriculture by providing high species specificity and may potentially lessen dependence on conventional pesticides with broader toxicity. Realizing the full potential of agricultural RNAi will depend on continuous technical improvements, harmonized regulatory processes, and effective outreach to educate and inform public and governmental stakeholders.

Research

The cellular uptake of dsRNA is a key hurdle to overcome when considering RNAi as a pest control solution. While much of the intercellular silencing mechanism is relatively conserved between organisms, barriers to dsRNA cellular entry can vary drastically, necessitating significant research into each pest species of concern. Characterization of dsRNA uptake in fungal pathogens is still at an early stage, and many questions related to RNA delivery, uptake, and efficacy in some economically important pest species remain despite technological advancements for other species. One key question pertains to the involvement of a systemic RNAi response (movement from cell to cell) in insect mortality. Further understanding of the mechanisms involved in systemic RNAi may inform approaches to enhance its activity in species refractory to RNAi. Another research objective necessary for widespread pesticidal dsRNA product development is devising efficient methods for effective gene target identification. While whole *genome* screens are less available in agricultural pests, data from screens in model insects sometimes translates to agricultural pests, providing useful data (Knorr et al. 2018). Developing approaches for whole *transcriptome* gene target knockdown screens in the pest species of concern would greatly aid in establishing target sensitivity when deciding the best gene candidates. Overall, knowledge of RNAi in plant pathogens is less developed than in insects. However, early successes point to significant potential to protect against plant pathogens.

Identifying genetic determinants for dsRNA resistance mechanisms is critical to guide resistance management practices and develop RNA technology to overcome or mitigate against resistance evolution. While currently available data suggest similarities in resistance phenotypes (Khajuria et al. 2018; Mishra et al. 2021), further information on whether distinct resistance mechanisms may evolve depending on the method of RNA delivery (*genetically modified* crops versus foliar sprays) is also needed. Identifying reduced uptake of dsRNA as a relevant mechanism of resistance (Khajuria et al. 2018) highlights the critical need for detailed knowledge of the dsRNA uptake process and potential strategies to improve it. Examples include the structural modification of the dsRNA to increase uptake (Abbasi et al. 2020), coupling of dsRNA to lipids and *nanoparticles* to reduce degradation by gut nucleases and accumulation in endosomes (internal vesicles) in Lepidoptera (Parsons et al. 2018: Gurusamy et al. 2020a; b). Novel dsRNA production and delivery methods, such as dsRNA-producing viruses and *symbionts* (Whitten et al. 2016), should be explored as they may help resolve some of the current delivery challenges presented by difficult-to-control pests. However, these approaches involve *genetic modification* technology that will open new regulatory questions.

The biosafety of nucleic acids has been established (US EPA 2001). The safety assessment of products based on dsRNA is based on the final formulation and end-use. Dependence on sequence complementarity supports predictability for lack of effects on non-target organisms through careful dsRNA design. Yet, risks of off-target effects (effects on genes that are not the target) and non-target organisms should be considered, and products designed to minimize potential effects. The development of *genomic* and *transcriptomic* resources should improve the accuracy of *in silico* analysis; however, bioinformatics alone is not predictive of dsRNA hazards. While environmental persistence of naked dsRNA appears short, effects of modifications to improve stability against degradation and evidence of long stability in some cases (San Miguel and Scott 2016) support risk assessments related to persistence on a case-by-case basis (Christiaens et al. 2018a; Bachman et al. 2020; Kleter 2020). Thus, while dsRNA molecules get adsorbed by soil particle surfaces and degrade in the environment, research on the persistence of formulated dsRNA formulation is appropriate in the context of specific problem statements. The risk due to oral uptake of environmental dsRNA by vertebrates and humans is considered

low based on the existence of chemical and physical barriers in the digestive tract and the history of safe exposure to dietary dsRNA. However, the effects of dsRNA formulated products on toxicity to vertebrates needs to continue to be considered in the context of the routes of exposure to inform safety evaluations (OECD 2023).

Barriers to Commercialization

Most GM plants are limited to nuclear transformation with the RNAi construct. For some crops, challenges remain for efficient delivery and precise integration of an RNAi construct into the plant *genome* and regeneration of plant lines following transformation. However, recent advances in generating and selecting transformed plants aim to improve the efficiency of *transgenic* plant production. Also, as described earlier, chloroplast-based dsRNA production may benefit certain future applications. However, *plastid* transformation largely relies on *gene gun* technology (particle biolistic) which remains expensive, inefficient, and difficult for most crop species.

Sprayable dsRNA and GM pesticidal products face practical challenges related to production cost and development time. In some cases, innovative, cost-efficient dsRNA production methods can overcome the cost of goods limitations for sprayable dsRNA delivered to plant foliage. However, some potential applications, such as soil drenches or trunk injections, might still be too expensive. Such cases represent opportunities for solutions aimed at improved formulation or modification of dsRNA that might reduce the amount of dsRNA needed to achieve an agronomic benefit.

The field-level efficacy of commercial dsRNA sprayable products is also crucial, and novel formulation and RNA structure design methods that allow for longer storage and activity periods are desirable. Greenhouse tests suggest high efficacy of dsRNA sprays against Colorado potato beetle, with plant protection detected after 14 (Rodrigues et al. 2021a) and up to 28 (San Miguel and Scott 2016) days after application. Reviews of field and greenhouse trials with dsRNA against lepidopteran larvae suggested variable stability of silencing effects (Xu et al., 2016), suggesting that the stability of dsRNA is likely variable and influenced by formulation, environmental conditions, crop, delivery method, and target pest physiology.

Product Perception

Understanding stakeholder questions and concerns is critical to the successful commercialization of RNAi-based products in agriculture. Stakeholder outreach and engagement allow for informed dialog and consumer education. Establishing the history of the safe use of dsRNA in crops by effectively communicating the safety profiles of approved traits and sprays will provide a foundation for advancing the understanding of RNAi of non-technical audiences. Results from consumer surveys have provided a starting point for understanding public perception of the new technology. While consumers in the United States, Canada, Australia, France, and Belgium were more willing to pay for products derived from grain produced using RNA sprays over *transgenic* Bt crops, the highest preference was for conventional products (Shew et al. 2016). Similar observations were reported for beef products produced using RNAi, although the extent of discount required varied substantially based on wording presented to consumers on the label (Britton and Tonsor 2019). Thus, there is a need to explain to the public that RNAi occurs naturally and that natural RNAs such as siRNAs and miRNAs are present in common foods like soybean, corn, and rice (Ivashuta et al. 2009; Petrick et al. 2013). Toxicology and safety testing required for registration by the US EPA assists with consumer education and increases confidence in the technology's safety. However, equivalent regulatory frameworks to assess dsRNA technology are lacking in some countries.

As more RNAi-based technologies become available, we will better understand public perception and whether risk perception differs between the various application methods. As more products are registered, it will be critical to have transparent, science-informed safety assessments and regulations to inform all stakeholders.

Abbreviations and Acronyms

bp	Base pair
Bt	*Bacillus thuringiensis*
CFIA	Canadian Food Inspection Agency
ckRNAi	Cross-kingdom RNA communication
DNA	Desoxyribonucleic acid
dsRNA	double-stranded RNA
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal, Insecticide, Fungicide, and Rodenticide Act
GM	Genetically Modified
HIGS	Host Induced Gene Silencing
MRL	maximum residue limits
mRNA	messenger RNA
miRNA	microRNA
nt	nucleotide
PTGS	Post-transcriptional gene silencing
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic acid
RNAi	RNA interference
OECD	Organization for Economic Co-operation and Development
SIGS	Spray Induced Gene Silencing
siRNA	small interfering RNA
sRNA	small RNA
TK-RNAi	Trans-kingdom RNAi
USDA	Unites States Department of Agriculture
VIGS	Virus Induced Gene Silencing

Glossary

Bioassays: method to determine the concentration or potency of a substance by its effects on living organisms, cells, or tissues.

Biotechnology: integration of biology and engineering to harness cellular and biomolecular processes to develop technologies and products to help improve human lives and the health of the environment.

Bioinformatics: an interdisciplinary field that develops computational and statistical techniques for comparing, analyzing, and interpreting biological data, particularly data sets that are large and complex.

Gene gun: biolistic particle delivery system used to deliver exogenous DNA, RNA, or protein to cells.

Gene Knockout: a genetic technique used to eliminate a gene or its function in an organism.

Genetically modified crop: Crop engineered to express traits using genetic materials from the same organism or a different organism (i.e., plant, insect, fungi, or bacteria).

Hemolymph: fluid in arthropods analogous to the blood in vertebrates that circulates in the body's interior and remains in direct contact with the animal tissues.

In silico: computational analysis.

Mutagenesis: the process by which an organism's DNA is changed by a mutation that can result in an alteration/loss in protein function and phenotypic changes. It may occur spontaneously in nature, from exposure to mutagens, or experimentally in the laboratory.

Nanocarrier: carrier system having a particle size <500 nm.

Nanoparticle: an ultrafine particle between 1 and 100 nanometers (nm) in diameter.

Nanosheet: two-dimensional nanostructure with thickness in a scale ranging from 1 to 100 nm.

Nuclease: an enzyme that cleaves chains of nucleotides in nucleic acids (i.e., DNA, RNA) into smaller units.

Phenotype: observable characteristics of an individual resulting from an interaction of its genes with the environment.

Plastid: membrane-bound organelle in plants, algae, and some non-plant eukaryotes cells. Examples include chloroplasts, chromoplasts, and leucoplasts.

Post-transcriptional gene silencing: a mechanism that degrades specific messenger RNAs, reducing gene expression (e.g., RNAi).

Sepaloid petals: flower petals that are green and look like sepals.

Spiracles: external respiratory openings that allow air to enter the trachea in insects for gas exchange.

Sublethal endpoints: measurement endpoints for sublethal effects that include development time, growth/weight, and reproduction that can be used to estimate population size.

Symbiont: an organism living in a close and prolonged interaction with an organism of a different species. Both organisms benefit from the exchange.

Transcript: RNA transcribed from a section of DNA.

Transcriptome: the complete set of messenger RNA (mRNA) an organism expresses.

Transgenic: an organism that contains DNA artificially introduced from an unrelated organism.

Transposon: DNA sequence that can change its position within a genome, altering a cell's genetic identity and genome size.

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