RNAi BASED PESTICIDES

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frontiers Research Topics

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RNAi BASED PESTICIDES

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EDITORIAL
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Editorial: RNAi Based Pesticides

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Editorial on the Research Topic

RNAi Based Pesticides

Development of new pesticide tools for farmers is needed to help increase food production. Approaches based on the use of nucleic acids triggering RNA silencing in plant pathogens and other pests in a sequence-specific manner are very promising and few preliminary works reported efficient protection of crops. Topically applied double stranded ribonucleic acids (dsRNAs)/small interfering ribonucleic acids (siRNAs) or spray-induced gene silencing (SIGS), also termed non-transformative RNAi technology to differentiate it from genetically modified (GM) plants designed to induce gene silencing through RNAi, are of particular interest.

On April 10-12, 2019, the Conference on RNAi Based Pesticides, held in Paris, France, supported by the Organisation for Economic Co-operation and Development (OECD)'s Co-operative Research Programme on Biological Resource Management for Sustainable Agricultural Systems, brought together academic and industry researchers, risk assessment experts, and environmental and food safety regulators, to discuss current research and policy issues related to this newly emerging, potential crop protection technology. The conference was attended by representatives from 14 OECD countries (Australia, Austria, Belgium, Canada, the Czech Republic, Denmark, Estonia, France, Germany, Hungary, the Netherlands, Switzerland, the United Kingdom, and the United States of America), and the European Food Safety Authority (EFSA). Gathering such a broad range of experts together allowed in-depth exploration of the possibilities of the application of external RNAs as pesticide active ingredients and discussions on the current state of knowledge and topics to help in developing considerations for risk assessment and corresponding foreseen government regulations. Two major questions addressed during discussions are (1) Are the current approaches to environmental and human health risk assessment of conventional and biological pesticides and or GM technologies applicable to the risk assessment of dsRNA based pesticides? and (2) Are additional data needed to be developed for dsRNA based pesticides?

On the basis of the conference presentations and expert discussions at the event, the present collection of research papers was launched as a joint Research Topic of the leading scientific periodicals, *Frontiers in Plant Science* and *Frontiers in Microbiology* to publish studies presented at the conference and submissions from experts working in the subject. Overall, 50 authors contributed 14 articles (2 original research articles, 5 reviews, 3 mini-reviews, and 4 perspective papers) discussing the possible utilization, but also rigorously considering the possible hazards and risks of external application of dsRNA molecules, their environmental fate, and effects possibly exerted on non-target organisms and human health.

THE MODE OF ACTION OF RNA BASED PESTICIDES

Several papers discuss the molecular biological mechanisms and other fundamental aspects of RNA-silencing. In his introductory overview on the mode of action of dsRNA induced

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sequence-specific RNA-silencing as a defense pathway in invertebrates and plants against viruses that produce dsRNA, Svoboda describes the main steps in the RNA degradation mediated by core protein components RNase III Dicer producing siRNA duplexes from dsRNA, endonuclease Argonaute, and RNA-dependent RNA polymerase. Besides outlining the molecular mechanism, he addresses the RNAi technology as a potent tool against agricultural pests but also warns about unintended off-target effects due to RNAi activity in species other than the pest organism aimed, about the possibility of transfer of small RNAs and RNAi among species and about the potential emergence of resistance to RNAi.

A detailed analysis of enzymes Argonaute and Dicer in a model grass plant the purple false brome (*Brachypodium distachyon*) is presented by Šečić et al.. Previously, various forms of the two enzyme families have been identified in the most common model organism in plant biology, the thale cress (*Arabidopsis thaliana*). This report identifies an expanded range, 16 members of the Argonaute family and 9 members of the Dicer family in *B. distachyon*, and also provides domain characterization, phylogenetic investigation supported by 3D protein modeling, as well as organ- or tissue-specific expression analysis of these proteins.

PESTICIDE ENVIRONMENTAL RISK ASSESSMENT AND MANAGEMENT

In most countries of the world, environmental risk assessment in the pesticide registration process is supported by non-target organism toxicity and environmental fate laboratory testing and, in some cases, field studies. Predicted environmental exposure concentrations (PECs) of the target compound(s) must not exceed given thresholds considered safe for non-target organisms, where these exist. Processes may differ internationally from region to region.

Pesticide registration in the European Union (EU) is completed in a dual process: the active ingredients are approved at EU level, and the formulated plant protection products are authorized at Member State levels. In the United States (US), the Environmental Protection Agency (EPA) requires registration of pesticide products and evaluates the pesticide active ingredient and formulated products. States or territories within the US require pesticide registration as well.

APPLICATION POSSIBILITIES OF RNA BASED PESTICIDES

The fundamental biochemical study by Šečić et al., mentioned above, in a model grass plant opens a knowledge base toward agronomically important cereals targeted by RNAi-based plant protection strategies e.g., barley or wheat. In addition, several other studies in the Research Topic mention various crop application promises. A direct possibility of application of external RNAi in crop protection is to spray dsRNA directed against pest-specific genes onto plants. Fungi or insects will take up these RNAs and process them to complex siRNA mixtures, which affect their survival or growth. Aspects of practical applications either against insect or plant pathogen microorganism pests from RNA delivery in water-soluble formulations (including foliar applications, trunk injection, and substance administration via irrigation) are reviewed by Cagliari et al. also summarizing successful application cases so far. The authors also compare the advantages of transformative (i.e., transgenic) and non-transformative (i.e., spray) RNAi applications.

Werner et al. illustrate RNAi applicability by assessing the efficacy of dsRNAs applied in SIGS in barley to suppress infestation by *Fusarium graminearum*, and verify effective gene silencing by measuring declines in the transcript levels of target genes in *F. graminearum* grown in the infected leaf tissue of the plant treated with different targeting dsRNA constructs. Interestingly, better RNAi effects were reached when manually designed dsRNA constructs (40–74% inhibition of gene expression) were used than in the case of computationally-designed constructs (44–52% inhibition of gene expression).

Different delivery strategies of RNAi-based products (i.e., dsRNA) for insect control are addressed by Christiaens et al.. They draw distinctions among host-induced, virus-induced, and spray-induced gene silencing (HIGS, VIGS, and SIGS). This review also summarizes field application difficulties e.g., physiological and cellular barriers leading to efficacy loss in insects, and advises novel non-transgenic delivery technologies e.g., polymer or liposomic nanoparticles, peptide-based delivery vehicles, and viral-like particles to overcome these barriers.

RISK ASSESSMENT OF RNA BASED PESTICIDES

RNAi can be utilized in crop protection either by plant-incorporated protectants through plant transformation (i.e., transgenic plants) or by non-transformative strategies through SIGS. Prior to the consideration of externally applied dsRNAs, GM plants designed to induce gene silencing through RNAi have already been developed and submitted as intended regulated products for authorization including food/feed safety assessment and environmental risk assessment. EFSA has already held an international scientific workshop and commissioned three external scientific reports on the subject, published an internal note on the strategy of off-target identification/prediction and risk assessment of RNAi based GM plants, as presented by Papadopoulou et al.. EFSA generally considers existing science-based risk assessment approaches for GM crops suitable also for RNAi-based GM plants, with certain specificities in the latter group. Another special form of transformative RNAi application that utilizes microbe- or virus-induced gene silencing also requires in-depth environmental assessment and falls under the regulation of GM organisms, as such methods involve release into the environment of viruses, bacteria, yeasts, or fungi genetically modified to act as a vector to generate RNAi induction by a continuous production of dsRNA into the host. Environmental decay or inactivation of the GM microbe- or virus vectors after an application is an issue in this field. The external application

of naked or formulated dsRNAs does not imply this aspect, as it does not involve the release of a live reproducing organism.

Bioinformatic analyses provide useful information for risk assessment, as non-target organisms with genes with some level of sequence homology to the gene intended for silencing in the target pest/pathogen can be identified by them, but the presence of RNAi activity cannot be reliably predicted in all representative non-target organisms, therefore, this approach cannot be used as a stand-alone tool. On the basis of the assessment of the above GM plants, Rodrigues and Petrick are of the opinion that the experience with the review of dsRNA-based GM crops has demonstrated that the existing regulatory paradigm for biologically based crop protection products is also adequate for the mode of action of externally applied dsRNA.

Environmental stability is a key issue in the use of dsRNAs. Bachman et al. emphasize that in order to provide environmental risk assessment and information on potential exposures, *in planta* produced (GM crops) and topically applied dsRNA (spray application), dsRNA must be successfully measured in relevant environmental compartments (soil, sediment, surface water). Unformulated dsRNAs were found to be highly labile (reported DT_{50} are in the range of 0.5–0.7 days), decomposed mostly by rapid microbial degradation of nucleic acids, but photodegradation and wash-off due to rain or dew also contribute to dissipation. Formulations can enhance dsRNA stability in the environment and can facilitate penetration through physical or biochemical barriers in target pests. Thus, formulations of dsRNA with layered double hydroxide nanosheets ("BioClay") or a shaped poly(2-(dimethylamino)ethyl acrylate) analog could achieve a 4-fold increase in its stability. BioClay formulation of dsRNAs is discussed in more detail by Fletcher et al..

Formulation impacts risk assessment for topically applied dsRNA, since certain formulants can substantially increase persistence. Exposure and hazard levels, therefore, have to be estimated considering the formulation, only taking the short half-life of the naked siRNA into account is not sufficient and would obviously be misleading. Therefore, information and/or studies on the impact on uptake and environmental persistence that the formulation presents are important to characterize exposure to the dsRNA. Where specific product formulations impact barriers to and uptake of the dsRNA, product-specific formulation toxicology testing on organisms or test surrogates would help better characterize the potential for hazard. The necessity of such testing depends on the legal requirements in the different OECD member countries as well as the characterization of the product.

Among the assessments presented in this Research Topic Fletcher et al., Neumeier and Meister, and Rodrigues and Petrick give utterance to the view that miRNA-like off-target activity of externally applied dsRNAs on various species (other than the pest) is negligible. Rodrigues and Petrick conceive that on the basis of exposures through different routes (ingestion, dermal absorption, inhalation), possible biological barriers, and the history of safe RNA consumption considered, harmful effects to humans are unlikely at dietary uptake level.

Oppert and Perkin propose a genome-wide expression analysis, termed RNAiSeq, in targeted pests (insects) to validate effective knockdown of target genes and to assess effects of possible knockdown on non-target genes by RNAi. Using this method on a coleopteran model insect the red flour beetle (*Tribolium castaneum*) they validated effective knockdown of various genes in several case studies e.g., a gene (*TC01101*) encoding the primary cysteine peptidase, a major digestive enzyme in *T. castaneum* larvae, and other genes encoding enzymes or other proteins involved in cuticle physiology.

In contrast, Raybould and Burns state that the establishment of any off-target effect inventory produced by profiling methods is unnecessary as such a fundamental research approach does not effectively support decision-making. The risk assessor only needs to take into consideration whether a dsRNA based agent presents acceptable or unacceptable risks. In their opinion methods for assessing exposure of and toxicity to non-target organisms by dsRNA based substances can be adapted from those being currently applied for chemical pesticides. They urge the use of targeted risk assessment on the basis of thresholds of unacceptability. If given externally applied dsRNAs do not pose unacceptable risk i.e., their toxicity:exposure ratio does not exceed a pre-set level, they should be approved. Romeis and Widmer allude to a somewhat similar approach, yet acknowledging the need for protection of certain non-target organisms in the agroecosystem, for example, those representing biodiversity protection goals of valued ecosystem services (Millennium Ecosystem Assessment [MEA], 2005). They consider an environmental risk assessment approach similar to that used in the case-by-case assessment of GM plants, but allowing flexibility to non-target risk assessment and being based on the selection of the most appropriate negative and positive control treatments suitable for externally applied RNAi based pesticides. In addition, they also emphasize the importance of the formulation in which dsRNAs are being applied (see above).

Strategies including RNA modifications or pooling of siRNAs to reduce off-target effects are outlined by Neumeier and Meister. Chemical modification (2′-*O*-methylation or incorporation of a locked nucleic acid) on the siRNA guide strands are reported capable of weakening the interaction between the guide strand and the target, and therefore, reducing miRNA-like off-target activities, without limiting siRNAs typically fully complementary to their on-target. Pooling at very low concentrations of multiple siRNAs directed against the same on-target at different positions but with different off-target signatures has also been indicated to reduce miRNA-like off-target effects.

EXPERT DISCUSSIONS ON THE TOPIC

Views and opinions expressed on the environmental fate of dsRNAs, as well as risk assessment on non-target organisms and human health during panel and overall discussions at the conference are summarized by Mendelsohn et al.. Key considerations from these conference discussions have already been incorporated into an OECD Working Document [OECD (Organisation for Economic Co-operation and Development),

2020] that facilitates regulators in evaluating externally applied dsRNA based products for potential environmental risks. Thus, diverse discourses regarding the use of dsRNA products in agriculture, regulatory, and risk assessment experience with dsRNA based products, and focused thematic issues, representing multiple perspectives, are reported. A definite intention of all scientific events sponsored by OECD CRP is to particularly address policy issues to help decision-makers to formulate official regulations better serve to support sustainable agriculture objectives. Therefore, such policy relevance has strongly been emphasized in the conference discussions. A consensus has been reached that current protocols used in hazard and risk assessment of pesticides have to be tailored for dsRNAs. Additionally, any evaluation of a dsRNA-based pesticide should include monitoring for degradation of the dsRNA over time. The importance of product formulation on environmental persistence of dsRNA and uptake by non-target organisms have been emphasized as topics that require special consideration. In addition, although health risks on humans and other mammals to environmental dsRNAs were deemed to possibly extendable to other vertebrates, it has been emphasized that current knowledge is insufficient to predict corresponding responsiveness across invertebrate taxa. For organisms that have been demonstrated to be responsive to environmental RNA, consideration of life cycle studies (growth, development, and reproduction) and studies on other non-lethal effects could be considered. A lacking thematic issue, unfortunately not substantially addressed during the conference, is the possibility of pest resistance development. We understand that this issue may also be considered.

AUTHOR CONTRIBUTIONS

AS and MM were an organizer and a section mediator at the OECD Conference on RNAi Based Pesticides and edited on behalf of the OECD conference contributions to this Research Topic. All authors were involved in writing this editorial and agree to its final version.

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ORIGINAL RESEARCH
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Further Elucidation of the Argonaute and Dicer Protein Families in the Model Grass Species *Brachypodium distachyon*

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RNA interference (RNAi) is a biological process in which small RNAs regulate gene silencing at the transcriptional or posttranscriptional level. The trigger for gene silencing is double-stranded RNA generated from an endogenous genomic locus or a foreign source, such as a transgene or virus. In addition to regulating endogenous gene expression, RNAi provides the mechanistic basis for small RNA-mediated communication between plant hosts and interacting pathogenic microbes, known as cross-kingdom RNAi. Two core protein components, Argonaute (AGO) and Dicer (DCL), are central to the RNAi machinery of eukaryotes. Plants encode for several copies of *AGO* and *DCL* genes; in *Arabidopsis thaliana*, the AGO protein family contains 10 members, and the DCL family contains four. Little is known about the conservation and specific roles of these proteins in monocotyledonous plants, which account for the most important food staples. Here, we utilized *in silico* tools to investigate the structure and related functions of AGO and DCL proteins from the model grass *Brachypodium distachyon*. Based on the presence of characteristic domains, 16 BdAGO- and 6 BdDCL-predicted proteins were identified. Phylogenetic analysis showed that both protein families were expanded in *Brachypodium* as compared with *Arabidopsis*. For BdDCL proteins, both plant species contain a single copy of DCL1 and DCL4; however, *Brachypodium* contains two copies each of DCL2 and DCL3. Members of the BdAGO family were placed in all three functional clades of AGO proteins previously described in *Arabidopsis*. The greatest expansion occurred in the AtAGO1/5/10 clade, which contains nine BdAGOs (BdAGO5/6/7/9/10/11/12/15/16). The catalytic tetrad of the AGO P-element-induced wimpy testis domain (PIWI), which is required for endonuclease activity, is conserved in most BdAGOs, with the exception of BdAGO1, which lacks the last D/H residue. Three-dimensional modeling of BdAGO proteins using tertiary structure prediction software supported the phylogenetic classification. We also predicted a provisional interactome network for BdAGOs, their localization within the cell, and organ/tissue-specific expression. Exploring the specifics of RNAi machinery proteins in a model grass species can serve as a proxy for agronomically important cereals such as barley and wheat, where the development of RNAi-based plant protection strategies is of great interest.

Keywords: protein, structure, prediction, RNAi, Argonaute, Dicer, *Brachypodium*

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INTRODUCTION

RNA interference (RNAi) is a regulatory mechanism utilized by most eukaryotes for endogenous gene silencing and protection against mobile repetitive sequences, transposons, and viruses (Fire et al., 1998; Wilson and Doudna, 2013). In contrast to transcriptional gene silencing (TGS), which results in the methylation of DNA and/or histones, posttranscriptional gene silencing (PTGS) operates by transcript degradation or translation inhibition. Selection of the target for silencing is governed by sequence complementarity between a single-stranded small RNA (sRNA) and the target RNA. Beyond its native role, the RNAi machinery has been exploited for developing novel plant protection strategies based on double-stranded (ds)RNA applications. Delivery of artificial dsRNA through transgene expression [host-induced gene silencing (HIGS)] or exogenous application [spray-induced gene silencing (SIGS)] was proven effective against fungal pathogens (Nowara et al., 2010; Koch et al., 2013; Koch et al., 2016; Wang et al., 2016), nematodes (Dutta et al., 2015), insects (Coleman et al., 2014; Abdellatef et al., 2015; Head et al., 2017), and parasitic plants (Tomilov et al., 2008; for review, see Andrade and Hunter, 2016; Cai et al., 2018). A recent discovery revealed that RNAi also is involved in natural cross-kingdom RNA communication (ckRNAi), where sRNA molecules function as mediators that are exchanged bidirectionally between a host plant and a microbial pathogen to silence their target transcripts and impact the outcome of the plant–pathogen interaction (Weiberg et al., 2013; Zhang et al., 2016; Wang et al., 2017a; Wang et al., 2017b).

Regardless of which RNAi-based process or application is involved, evolutionarily conserved protein components, including Dicer [termed Dicer-like (DCL) in plants] and Argonaute (AGO), play key roles in dsRNA processing. Dicers and DCLs are RNase III endonucleases that process exogenously supplied or endogenously generated ds- or hairpin (hp)-containing RNA precursors into various species of dsRNAs, commonly 21–24 nucleotides (nt) in length. These sRNAs are then loaded onto specific AGO proteins, which are components of the RNA-induced silencing complex (RISC). The loaded sRNA is processed into a single-stranded sRNA molecule, which then guides the RISC to complementary targets in the cytoplasm or nucleus. Depending on the biological context, target recognition leads to PTGS *via* RNA degradation, which may be mediated by the AGO protein's slicer activity, or inhibition of translation, or to TGS *via* genomic DNA and/or histone methylation (Carthew and Sontheimer, 2009; Poulsen et al., 2013; Borges and Martienssen, 2015; Fang and Qi, 2016).

Phylogenetic analysis of genes belonging to the Dicer family suggests that they arose early in the evolution of eukaryotes and that their duplication and diversification correlated with the development of multicellularity and the need for complex gene regulation (Mukherjee et al., 2013). In plants, the structure and function of DCL proteins have been investigated most intensively in *Arabidopsis*, which expresses four DCLs (Schauer et al., 2002). The domain architecture of these proteins, like that of other eukaryotic Dicers, generally consists of an amino-terminal DEXDc and helicase-C (HELICc) domain, which are thought to mediate processive movement along a dsRNA, a dicer-dimer (heterodimerization) domain that facilitates binding with protein partners (Qin et al., 2010), a P-element-induced wimpy testis (PIWI)–Argonaute–Zwille (PAZ) domain, which binds the 3' end of the dsRNA, two RIBOc (ribonuclease III family) domains and at least one dsRNA-binding motif (DSRM) domain at the C terminus (Schauer et al., 2002; Mukherjee et al., 2013; Bologna and Voinnet, 2014; Song and Rossi, 2017).

Analyses of *Arabidopsis* mutants revealed that the four DCLs generate different types of sRNAs, although some functional redundancy was observed (Gasciolli et al., 2005; Bologna and Voinnet, 2014; Borges and Martienssen, 2015). AtDCL1 produces microRNAs (miRNAs), a class of sRNAs that regulates endogenous gene expression via PTGS (Bartel, 2004). The remaining AtDCLs generate various subclasses of small interfering RNAs (siRNAs), including i) natural-antisense-transcript (nat)-siRNAs generated by AtDCL2 (Borsani et al., 2005), ii) trans-activating (ta)-siRNAs produced by AtDCL4 (Dunoyer et al., 2005), and iii) TGS-related 24-nt siRNAs generated by AtDCL3, which are responsible for silencing transposons and other repeated DNA sequences (Xie et al., 2004).

Despite the diversity of sRNAs, their association with AGO proteins and the RISC complex is a common feature. AGO proteins were named after the tube-shaped leaves of *Arabidopsis ago1* mutants, which resemble the tentacles of the pelagic octopus, *Argonauta argo* (Bohmert et al., 1998). AGO proteins are highly conserved in nature, although the size of this family varies substantially between species (Höck and Meister, 2008; Zhang et al., 2015; Fang and Qi, 2016; You et al., 2017).

AGOs have a high level of structure and domain conservation between the prokaryotic and eukaryotic variants, even when the biological function clearly differs (Willkomm et al., 2015). Several prokaryotic (Wang et al., 2009; Liu et al., 2018) and eukaryotic (Lingel et al., 2003; Lingel et al., 2004; Boland et al., 2011) complete AGO structures or individual domains have been crystallographically resolved. Several human AGO proteins have been crystallized, namely, AGO2 in complex with a miRNA (Elkayam et al., 2012), AGO1 (Faehnle et al., 2013), and AGO3 (Park et al., 2017), showing that the AGO activity is dependent on conservation of active site residues and their interaction with other protein regions. The structures of AtAGO proteins have been partly resolved, especially the middle (MID) domain of AtAGO1, AtAGO2, and AtAGO5 (Frank et al., 2012; Zha et al., 2012). Functional domains characteristic of all AGO proteins, including the *Arabidopsis* AGOs, are the PAZ, MID, and PIWI domains governing the binding of sRNA ends and the slicer activity (Höck and Meister, 2008; Frank et al., 2012).

In *Arabidopsis*, 10 different AGOs have been identified. Phylogenetic analyses have divided them into three clades, comprising AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 (Vaucheret, 2008). The different clades contain AGOs that mediate PTGS or TGS after they load specific types of sRNAs, which are selected based on length and identity of the 5' nt (Bologna and Voinnet, 2014; Zhang et al., 2015; Fang and Qi, 2016). For example, AtAGO1 is involved in endogenous developmental regulation by miRNAs (Vaucheret et al., 2004), antiviral defense (alongside AtAGO2, Harvey et al., 2011), as well as bidirectional ckRNAi

(Weiberg et al., 2013). AtAGO4 and AtAGO6 are involved in DNA and histone methylation (Zilberman et al., 2003; Zheng et al., 2007). AtAGO9 is known to be involved in female gametogenesis (Olmedo-Monfil et al., 2010), while AtAGO10 competes with AtAGO1 for sRNA loading in regulation of shoot apical meristem development (Zhu et al., 2011). AtAGO7 plays a role in defense against viruses (Qu et al., 2008).

In comparison to *Arabidopsis*, little is known about the RNAi machinery components in monocots. The number of AGO proteins is expanded in cereals, as there are 17 AGOs in maize (Zea mays) and 19 in rice (Oryza sativa; Fang and Qi, 2016; Mirzaei et al., 2014; Patel et al., 2018). The copy number of DCL2 and/or DCL3 genes also differs between monocot species and *Arabidopsis*. Six predicted DCLs were identified in rice, while five were identified in maize, wheat, and barley (Margis et al., 2006). Furthermore, the DCL3b gene has diverged significantly from its DCL3a paralog (Margis et al., 2006) and, thus, is considered a distinct, monocot-specific class of Dicer, termed DCL5 (Fukudome and Fukuhara, 2017; Borges and Martienssen, 2015).

Cereals are major staple crops worldwide; however, a plethora of pathogens and pests threaten their production (Savary et al., 2012). Recent efforts to develop environmentally friendly plant protection strategies have demonstrated that HIGS and SIGS can be used in major cereal crops, such as barley and wheat, to control necrotrophic fungi (Koch et al., 2013; Koch et al., 2016; Koch et al., 2018) and aphid pests (Abdellatef et al., 2015). Developing a better understanding of the cereal AGO and DCL protein family members and their specific functions is a prerequisite for clarifying the mechanisms undergirding RNAi-mediated plant protection. Here, we use the model species for temperate grass plants, *Brachypodium distachyon* (*Brachypodium*), to investigate cereal AGO and DCL proteins. *Brachypodium* is self-fertile, has a small genome (~272 Mb), a short life cycle, and established transformation protocols (Vogel et al., 2006). The commonly used diploid inbred line Bd21 is fully sequenced (The International Brachypodium Initiative, 2010). In addition, literature data reveal strong responsiveness of *Brachypodium* sRNA pools to abiotic stress, suggesting that the RNAi machinery is sensitive to environmental changes (Wang et al., 2015).

Based on genomic database searches and *in silico* analysis, we identified six BdDCL proteins, as well as 16 previously reported AGO protein sequences in *Brachypodium* (Mirzaei et al., 2014). Since the structure of proteins closely relates to function and thus can serve as an indication of interaction patterns and redundancy in large protein families, we especially looked into domain structure conservation in *Brachypodium* relative to *Arabidopsis*. Similar to the protein structure and interactome analysis applied in Secic et al. (2015), we subjected Bd AGO and DCL proteins to a series of *in silico* analysis steps. The focus of this study is the structures and related functions of the AGO-like and DCL proteins of *B. distachyon*, with special regard to analysis of the phylogeny and three-dimensional (3D) structure modeling of the AGO family, as compared with the more familiar *Arabidopsis thaliana* AGO protein family. Given that the At AGO1/5/10 clade contains proteins involved in ckRNAi, we were especially interested to define the BdAGO proteins that are structurally most related to this clade and thus potentially have a key function in plant immunity and RNAi-based plant protection.

MATERIALS AND METHODS

Acquisition of Sequences and Database Search

AGO and DCL protein sequences corresponding to the primary transcripts of specific genes were acquired by searching the Plant Comparative Genomics portal Phytozome 12 (Goodstein et al., 2012) *B. distachyon* v3.1 database (The International Brachypodium Initiative, 2010). Proteins whose domain architecture resembled those of *Arabidopsis* AGO and DCL proteins were considered. The *Arabidopsis* AGO and DCL protein sequences were taken from The *Arabidopsis* Information Resource database (Rhee et al., 2003; Berardini et al., 2015). Information on resolved protein structures was acquired from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (Berman et al., 2000; Burley et al., 2018). The Brachypodium eFP Browser (Sibout et al., 2017; Winter et al., 2007) was used to assess the expression of transcripts corresponding to proteins involved in this study, based on the expression atlas detailing different organs and developmental stages.

Phylogenetic Analysis, Interactome Analysis, and Localization

The phylogenetic analysis and tree rendering were done by the Phylogeny.fr web server (Dereeper et al., 2008; Dereeper et al., 2010). The operational sequence is composed of MUSCLE 3.8.31 (Edgar, 2004) for alignment with default settings, Gblocks 0.91b for removal of ambiguous regions (Castresana, 2000), PhyML 3.1/3.0 aLRT for phylogeny (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006), based on maximum likelihood, and TreeDyn 198.3 for graphical representation (Chevenet et al., 2006). Multiple sequence alignment (MSA) was done using Clustal Omega at European Molecular Biology Laboratory—European Bioinformatics Institute (Sievers et al., 2011; Goujon et al., 2010) and the conserved residues and domains visualized by the Mview multiple alignment viewer (Brown et al., 1998). Pairwise sequence alignments were done using EMBOSS Needle (Rice et al., 2000), utilizing the Needleman–Wunsch algorithm for global alignment. Domain search was conducted using Simple Modular Architecture Research Tool (SMART) in normal SMART mode (Schultz et al., 1998; Letunic and Bork, 2017) and visualized with the Illustrator for Biological Sequences (IBS) online illustrator (Liu et al., 2015). Prediction of protein location was done using the plant subcellular localization integrative predictor (PSI), which shows an integrative result based on the output of an 11-member predictor community (Liu et al., 2013). Prediction of the interactome was done using the STRING database of protein–protein associations, while searching by protein

sequence (Szklarczyk et al., 2019). Resulting associations/possible interactions that originate from text mining have been excluded, and the results show only associations supported by co-expression and/or experimental data.

Three-Dimensional Structure Modeling and Validation

SWISS-MODEL, a homology-based modeling software available at the ExPASy web server (Waterhouse et al., 2018), and CPHmodels 3.2 protein homology modeling server (Nielsen et al., 2010) were both used for 3D structure prediction from the sequence data. BLAST (Camacho et al., 2009) and HHBlits (Remmert et al., 2011) template search through the SWISS-MODEL template library was done and the models built using ProMod3 (Waterhouse et al., 2018) and the target-template alignment.

QMEAN, used for validation of the predicted 3D structures, is a scoring function that considers single residues and the global model, delivering an estimation of absolute quality of the prediction (Benkert et al., 2011). In order to check the stereochemical quality of predicted structures, we used the PROCHECK program (Morris et al., 1992; Laskowski et al., 1993). One of the stereochemical parameters considered is the fitness of the model in a Ramachandran plot, which maps the allowed backbone dihedral angles of amino acids (aa) in a protein structure (Ramachandran et al., 1963). Further on, we used the WHATCHECK software (Hooft et al., 1996) to calculate the Ramachandran Z-score, which compares the quality of the query structure to structures with high confidence (Hooft et al., 1997). Lastly, we used the dDFIRE/DFIRE2 energy calculation (Yang and Zhou, 2008) to calculate free energy scores for our structure predictions.

PyMOL (The Py-MOL Molecular Graphics System) was used for visualization of the predicted structures (Schrödinger, 2010, Open-Source PyMOL 1.3).

RESULTS

Argonaute and Dicer Protein Families Are Expanded in *Brachypodium* Relative to *Arabidopsis*

To identify AGO and DCL proteins, the *B. distachyon* v3.1 database (The International Brachypodium Initiative, 2010) was searched for transcripts whose encoded proteins contain the characteristic domain architecture of each protein family. The accession numbers of the acquired sequences, the names assigned to the corresponding BdAGO proteins, the location of the encoding genes, and a description of the primary transcripts are shown in **Table 1**. The naming convention is similar to that used by Mirzaei et al. (2014) for 16 AGO proteins identified by primary transcripts in the *B. distachyon* Bd21 v3.1 annotation (The International Brachypodium Initiative, 2010). Our search for BdDCL candidates within the Bd21 v3.1 database revealed nine sequences. Clear lack of functional domains or insufficient length of the deduced aa sequence reduced the number of putative *DCL* genes to six (Bradi1g15440, Bradi1g77087, Bradi2g23187, Bradi5g15337, Bradi1g21030, and Bradi3g29287). Accession numbers and assigned names for the encoded BdDCLs are shown in **Table 2**. The putative AtAGO and AtDCL protein sequences were downloaded from The *Arabidopsis* Information Resource database (**Table S1**) and included in the MSA and phylogenetic analysis.

A phylogenetic analysis of the inferred BdAGO protein sequences relative to those of *Arabidopsis* AGOs is shown in **Figure 1**. BdAGO proteins were placed in all three AtAGO clades. Some were grouped with a specific AtAGO member within a clade (e.g., BdAGO8 was grouped with AtAGO7, and BdAGO5/6/7/10 were grouped with AtAGO5), whereas other BdAGOs were distributed throughout an entire clade (e.g., BdAGO1/2/3/4 within the AtAGO4/6/8/9 clade). In the AtAGO1/5/10 clade, BdAGO9/11/12/15/16 were interspersed

TABLE 1 | Assigned names and accession numbers of BdAGO proteins as well as genomic location and description of the primary transcript (as acquired from Phytozome Bd21 v3.1 database).

Assigned name of protein	Primary transcript ID (Phytozome)	Location	Description (Phytozome)
BdAGO1	Bradi2g10360.2	Bd2:8611187.8615652 reverse	PTHR22891//PTHR22891:SF44 – Eukaryotic translation initiation factor 2C
BdAGO2	Bradi2g14147.1	Bd2:12806099.12812784 reverse	PTHR22891:SF20 – Protein AGO 4-related
BdAGO3	Bradi2g10370.1	Bd2:8620394.8628745 reverse	AGO family, subfamily AGO4
BdAGO4	Bradi4g08587.1	Bd4:7715921.7724879 reverse	PTHR22891:SF35 – Protein AGO 6
BdAGO5	Bradi1g12431.2	Bd1:9307067.9313002 forward	PTHR22891//PTHR22891:SF49 – Eukaryotic translation initiation factor 2C
BdAGO6	Bradi1g05162.2	Bd1:3447373.3455769 forward	PTHR22891//PTHR22891:SF24 – Eukaryotic translation initiation factor 2C
BdAGO7	Bradi1g28260.3	Bd1:23482384.23489131 reverse	AGO family, subfamily monocot-AGO1
BdAGO8	Bradi1g16060.3	Bd1:12986117.12991032 reverse	AGO family, subfamily AGO7
BdAGO9	Bradi1g36907.2	Bd1:32760045.32772130 reverse	PTHR22891//PTHR22891:SF25 – Eukaryotic translation initiation factor 2C
BdAGO10	Bradi1g54977.1	Bd1:53536162.53543236 forward	PTHR22891//PTHR22891:SF36 – Eukaryotic translation initiation factor 2C
BdAGO11	Bradi1g29577.1	Bd1:25162908.25171156 reverse	PTHR22891//PTHR22891:SF57 – Eukaryotic translation initiation factor 2C
BdAGO12	Bradi5g18540.1	Bd5:21720455.21728815 reverse	AGO family, subfamily AGO1
BdAGO13	Bradi5g21810.1	Bd5:24487261.24492250 forward	AGO family, subfamily AGO2/3
BdAGO14	Bradi5g21800.1	Bd5:24479944.24484383 forward	AGO family, subfamily AGO2/3
BdAGO15	Bradi3g51077.3	Bd3:51944662.51956527 forward	PTHR22891//PTHR22891:SF34 – Eukaryotic translation initiation factor 2C
BdAGO16	Bradi3g60697.5	Bd3:59325332.59333596 reverse	PTHR22891//PTHR22891:SF34 – Eukaryotic translation initiation factor 2C

TABLE 2 | Assigned names and accession numbers of BdDCL proteins as well as genomic location and description of the primary transcript (as acquired from Phytozome Bd21 v3.1 database).

Assigned name of protein	Primary transcript ID (Phytozome)	Location	Description (Phytozome)
BdDCL1	Bradi1g77087.1	Bd1:73701094.73713218 forward	PTHR14950:SF3 – ENDORIBONUCLEASE DICER HOMOLOG 1
BdDCL2a	Bradi1g15440.1	Bd1:12353426.12376799 forward	DCL family, subfamily DCL2
BdDCL2b	Bradi1g21030.3	Bd1:16934990.16948923 reverse	PTHR14950:SF19 - ENDORIBONUCLEASE DICER HOMOLOG 2
BdDCL3a	Bradi3g29287.1	Bd3:31008845.31020951 forward	PTHR14950//PTHR14950:SF31 - HELICASE-RELATED//SUBFAMILY NOT NAMED
BdDCL3b	Bradi2g23187.3	Bd2:20726122.20733365 reverse	PF00636//PF02170//PF03368 – Ribonuclease III domain (Ribonuclease_3)//PAZ domain (PAZ)//Dicer dimerization domain (Dicer_dimer)
BdDCL4	Bradi5g15337.3	Bd5:18845215.18867304 reverse	PTHR14950:SF15 – DCL 4

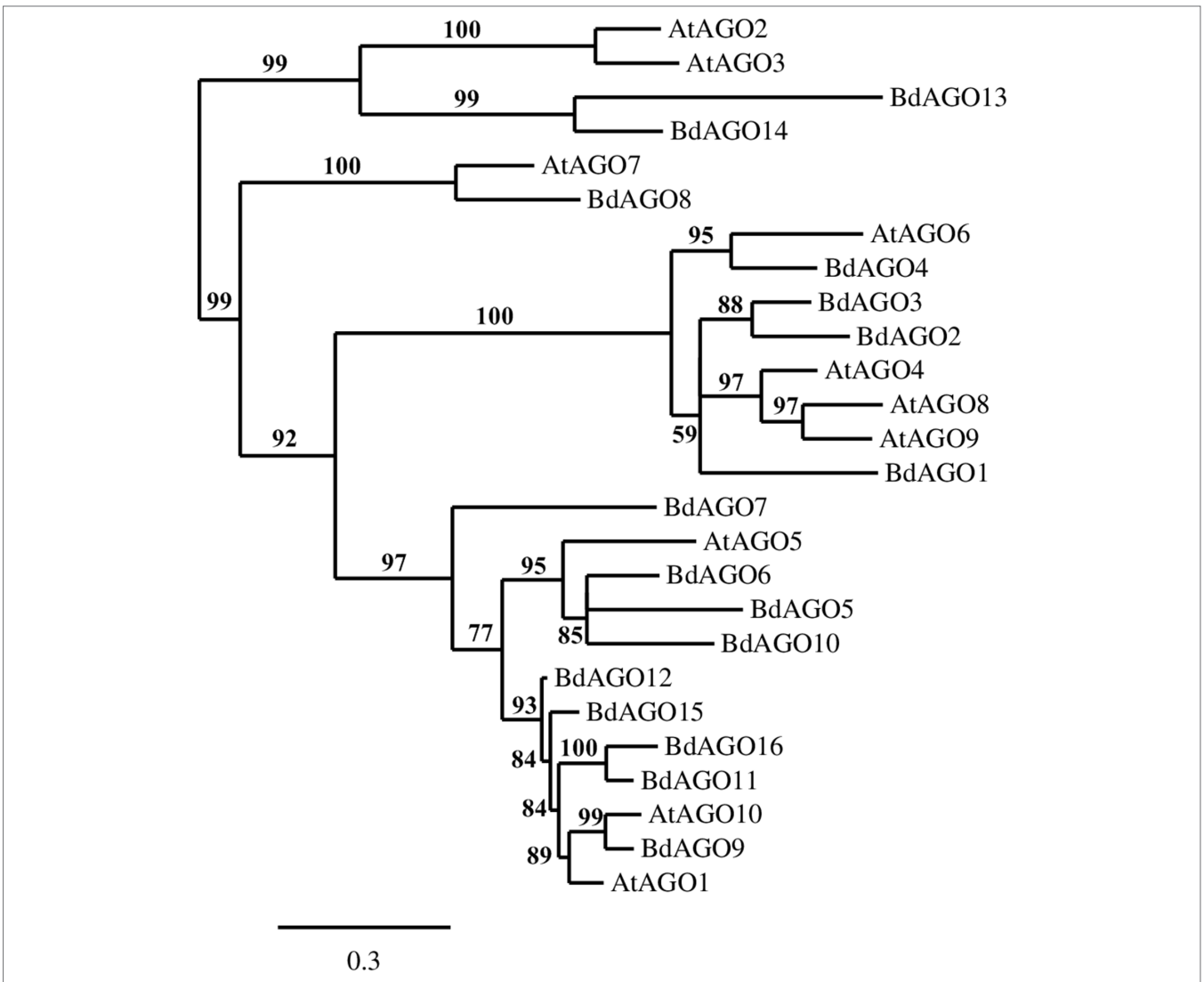

FIGURE 1 | Phylogram of the BdAGO and AtAGO protein sequences, as calculated by Phylogeny.fr (MUSCLE, Gblocks, PhyML, TreeDyn). Branch support values are displayed in percentages, and branch support values smaller than 50% are collapsed. Scale bar defining the branch length displayed in bottom right corner.

with AtAGO1 and AtAGO10. These findings suggest that the structural and functional differences of AtAGO proteins are translated to the expanded *Brachypodium* family. Phylogenetic analysis of the inferred BdDCL proteins showed that they strongly aligned with individual members of the *Arabidopsis* DCL family (**Figure 2**). Like *Arabidopsis*, *Brachypodium* contains a single ortholog of DCL1 and DCL4; however, expansion of the BdDCL family has led to the presence of two copies of both

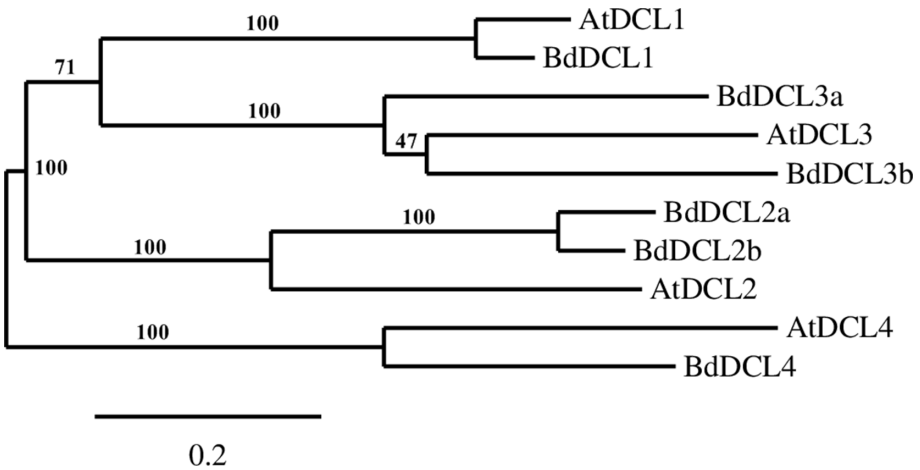

FIGURE 2 | Phylogram of the BdDCL and AtDCL protein sequences, as calculated by Phylogeny.fr (MUSCLE, Gblocks, PhyML, TreeDyn). Branch support values are displayed in percentages, and branch support values smaller than 50% are collapsed. Scale bar defining the branch length displayed in bottom right corner.

DCL2 and DCL3, as compared with the single ortholog present in *Arabidopsis*. Sequence comparisons revealed that DCL2a and DCL2b share 82.5% similarity at the aa level, while BdDCL3a and BdDCL3b share 44.2% similarity (aa, global alignment). Together, the phylogenetic trees show distinct branches interspersing *Arabidopsis* and *Brachypodium* homologues in functional clades; to our knowledge, this is the first indication of how the expansion of AGO and DCL protein families in *Brachypodium* relates to the specific clades and/or functional diversity of the corresponding *Arabidopsis* proteins.

Predicted Domains of BdAGO and BdDCL Proteins Indicate Structure Conservation

Next, we executed a domain search using SMART to elucidate the structures and functions of the 16 BdAGO proteins and six BdDCLs. The domain structure visualization of BdAGO (**Figure 3**) and BdDCL (**Figure 4**) proteins highlights the differences between members of each protein family with respect to the positions and presence/absence of the typically conserved domains. Detailed domain prediction data, as acquired by SMART/Pfam search, and the corresponding confidence values

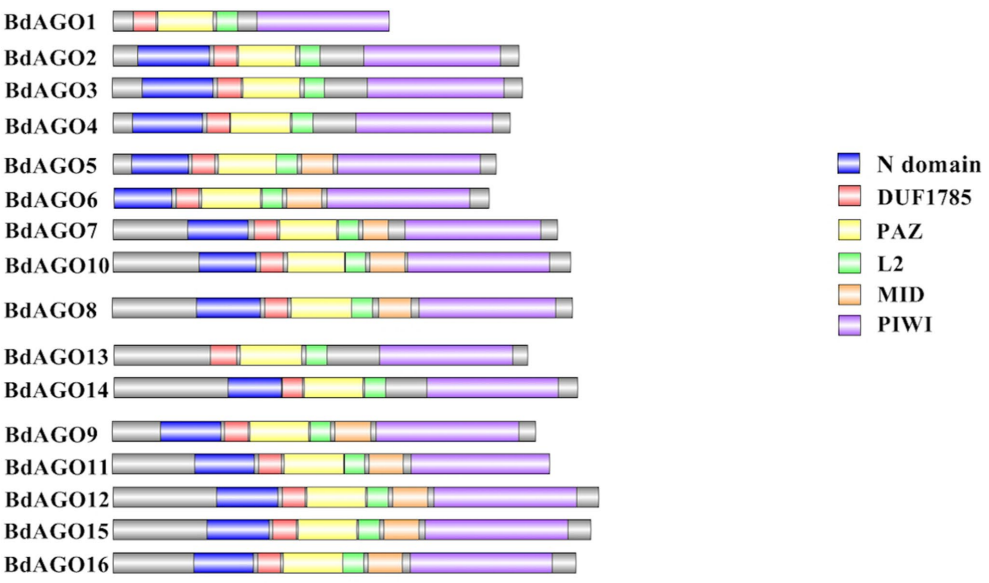

FIGURE 3 | Visual representation of domain structure of BdAGO proteins, as identified by domain search by SMART and Pfam. Picture generated with Illustrator for Biological Sequences illustrator. Displayed domains: N-domain, DUF1785 (L1), PAZ (PIWI Argonaut and Zwille), L2, MID, PIWI, sequence, with no domain predicted in gray.

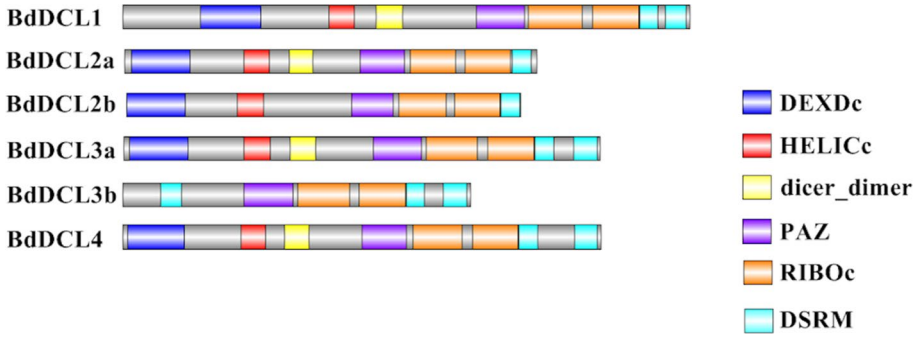

FIGURE 4 | Visual representation of domain structure of Bd DCL proteins, as identified by domain search by SMART and Pfam. Picture generated with Illustrator for Biological Sequences illustrator. Displayed domains: DEAD-like helicase superfamily (DEXDc), helicase superfamily c-terminal domain (HELICc), dicer_dimer, PIWI Argonaut and Zwille (PAZ), ribonuclease III family (RIBOc), double-stranded RNA-binding motif (DSRM), sequence with no domain predicted in gray.

are shown for BdAGO (**Table S2**) and BdDCL proteins (**Table S3**). Consistent with other eukaryotic AGO proteins, many members of the BdAGO family are predicted to have four characteristic functional domains, including the N-terminal domain, PAZ, MID, and PIWI domain (Zhang et al., 2014). However, while the domain prediction results identified a variable N-t domain in most BdAGOs that consisted of both an N-domain and a DUF1785 domain (Poulsen et al., 2013), BdAGO1 and BdAGO13 contained only the DUF1785 domain. In addition, BdAGO1, BdAGO2, BdAGO3, BdAGO4, BdAGO13, and BdAGO14 were not predicted to contain a MID domain, in comparison to a previous report (Mirzaei et al., 2014). MSA performed by Clustal Omega on the PIWI domain of BdAGO proteins (**Figure 5**) showed a typical pattern of conservation for the DEDD/H catalytic tetrad required for slicer activity and a conserved QF-V motif in all aligned sequences except BdAGO1, which has the

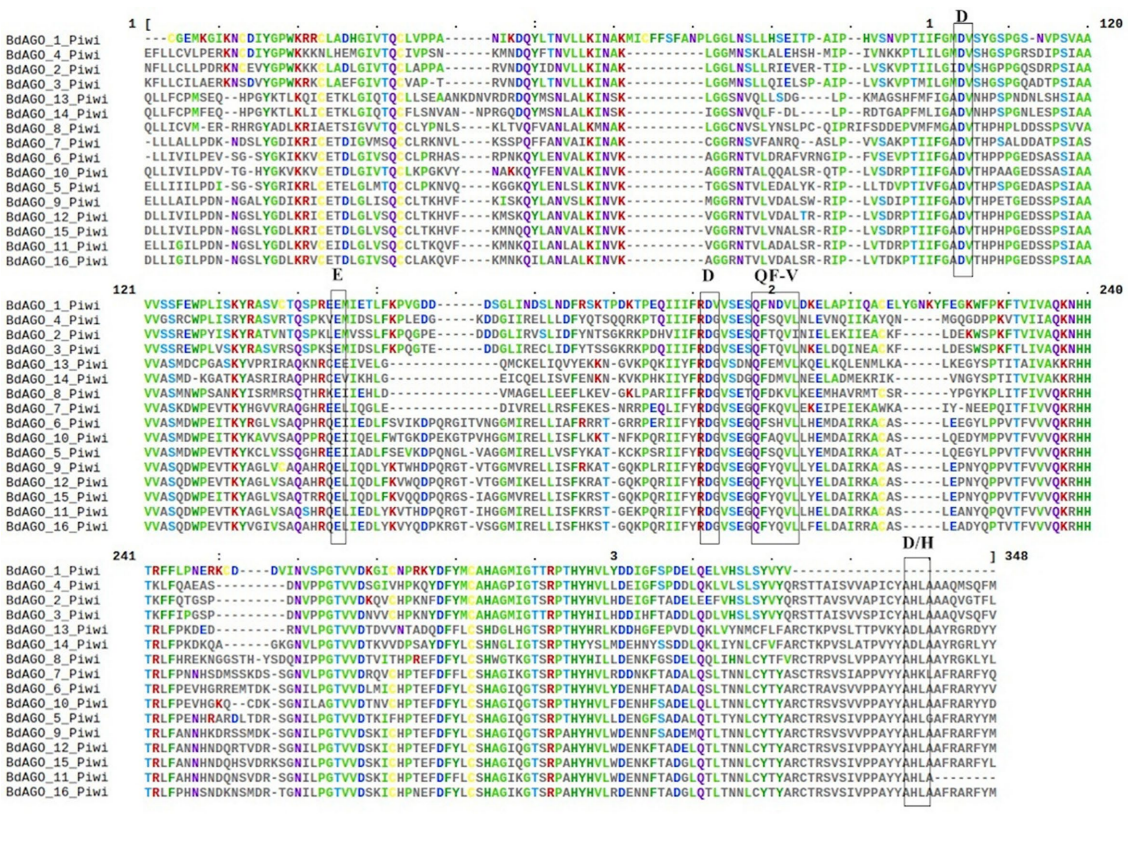

FIGURE 5 | Multiple sequence alignment (MSA) of the PIWI domain of BdAGO proteins, as acquired by Clustal Omega and Mview visualization. The catalytic tetrad DEDD/H (Asp-Glu-Asp-Asp or Asp-Glu-Asp-His) and the QF-V (Gln-Phe-Val) motifs are boxed.

shortest protein sequence of all the BdAGO proteins and lacks the D/H residue of the catalytic tetrad.

Analysis of the predicted domains in BdDCL proteins revealed that the characteristic DEXDc, HELICc, Dicer-dimer (DUF283, Qin et al., 2010), PAZ, RIBOc, and DSRM domains are present in most family members. However, BdDCL2b and BdDCL3b lack the dimerization domain; BdDCL3b additionally lacks both the DEXDc and HELICc domains and instead contains an additional DSRM domain at the N terminus (position: 131-218, E-value: 8.6e-17; **Figure 4** and **Table S3**). By contrast, BdDCL2a and BdDCL2b contain only one DSRM domain (**Figure 4**, **Table S3**).

Three-Dimensional Modeling Supports Phylogenetic Data Showing a Strong Expansion in the BdAGOs in the AGO1/5/10-Related Clade

In order to obtain an optimal homology-based 3D model of the studied proteins, we used SWISS-MODEL and CPHmodels 3.2. When choosing between models generated by alternative software programs or based on different templates, validation of the predicted structures is crucial for generating a consensus on the optimal model and further comparison. In case of BdAGOs, validation of the predicted structures was done using four different measurements, the results of which are shown in **Table S4**. While a 0-1 QMEAN value gives an absolute scoring of the predicted model, the Z-score shown in **Table S4** serves as a comparison of the quality of the prediction of the query model relative to expected from a high-resolution X-ray crystallography structure. Typically, the more negative the Z-score is, the lower the quality of the predicted structure. Using PROCHECK, we report on the percentage of residues that fall into the most favored regions of the Ramachandran plot. The free energy score of the conformation of the predicted protein calculated by dDFIRE usually indicates lower values for a better model. Based on validation of the 3D models by the software, SWISS-MODEL was chosen as the preferred modeling tool for BdAGO proteins (**Table S4**). The corresponding AtAGO 3D structures, predicted and validated in the same fashion (**Table S5**), were subsequently used alongside the visualization of the BdAGO proteins by PyMOL. **Figures 6**, **7**, and **S1** display the models, in which the PAZ, MID, and PIWI domains (where predicted) and residues comprising the DEDD/H catalytic tetrad are indicated for all BdAGOs and a corresponding AtAGO representing the appropriate branch of the phylogenetic tree depicted in **Figure 1**. Overall, the predicted structures of the BdAGOs mirror the

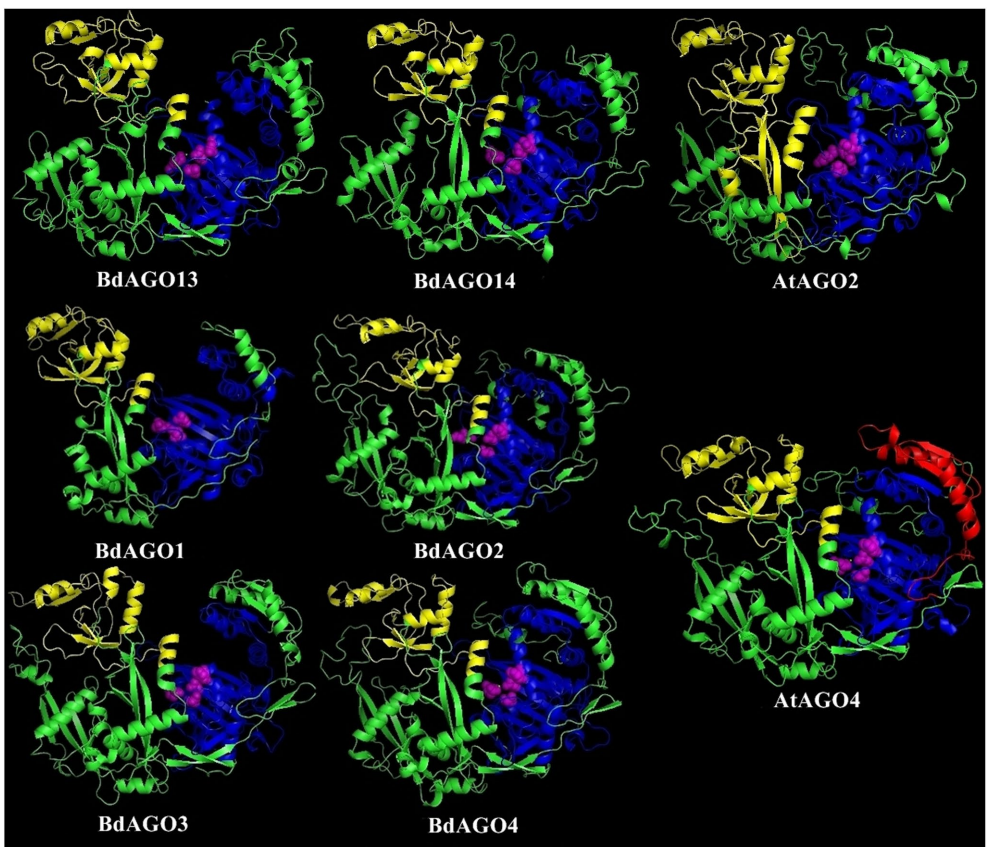

FIGURE 6 | Three-dimensional structure predictions for BdAGO13 and BdAGO14 (with AtAGO2 as the closest homolog in *Arabidopsis*) and BdAGO1, BdAGO2, BdAGO3, BdAGO4 (with AtAGO4 as the closest homolog in *Arabidopsis*), as modeled by SWISS-MODEL. PAZ (yellow), PIWI (blue), and MID (red) domains as predicted by SMART and Pfam displayed. The catalytic tetrad within the PIWI domain (DEDD) is marked by magenta spheres. Visualization by PyMOL.

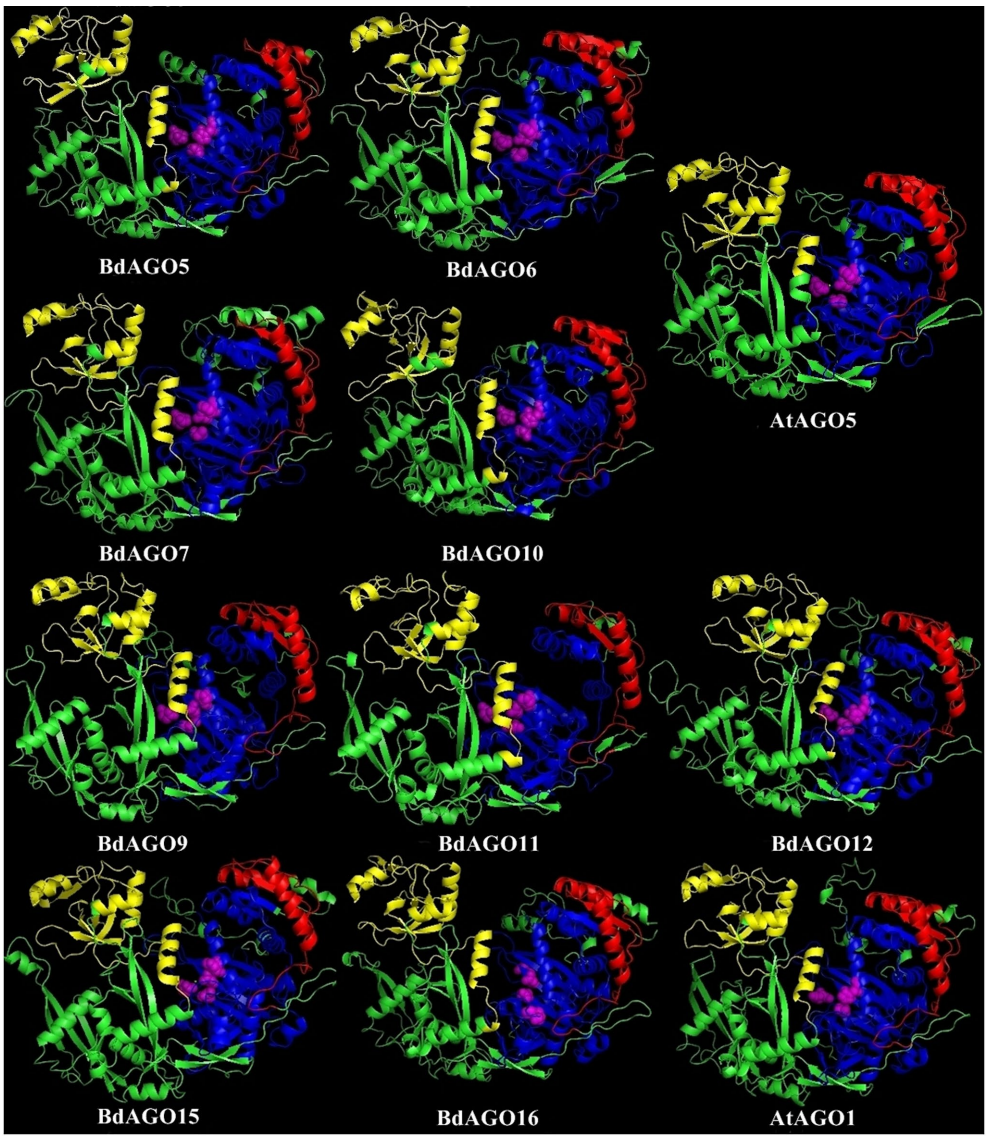

FIGURE 7 | Three-dimensional structure predictions for BdAGO5, BdAGO6, BdAGO7, and BdAGO10 (with AtAGO5 as the closest homolog in *Arabidopsis*) and BdAGO9, BdAGO11, BdAGO12, BdAGO15, and BdAGO16 (with AtAGO1 as the closest homolog in *Arabidopsis*), as modeled by SWISS-MODEL. PAZ (yellow), PIWI (blue), and MID (red) domains as predicted by SMART and Pfam displayed. The catalytic tetrad within the PIWI domain (DEDD) is marked by magenta spheres. Visualization by PyMOL.

corresponding AtAGO structures, suggesting a functional conservation. The PIWI domain and the catalytic tetrad especially show similarity between the clade members shown together in **Figures 6** and **7**. The BdDCL proteins did not have successfully modeled structures predicted by either software and thus are not shown.

Expression Analysis and Putative Interactors of BdAGO Proteins

We addressed the question of tissue-specific expression of *BdAGO* and *BdDCL* genes by utilizing the *B. distachyon* eFP Browser (Sibout et al., 2017; Winter et al., 2007) in **Table 3**. Stronger expression of *BdAGO* and *BdDCL* genes was observed in seed and stem tissue compared with roots or leaves. The plant subcellular localization integrative predictor used for protein localization predicted that all BdAGOs reside in the cytosol, except for BdAGO3, BdAGO14 (predicted to localize in the nucleus), and BdAGO7 (predicted to localize in plastids), with varying scores of confidence (**Table S7**).

Finally, prediction of proteins that interact with BdAGOs was carried out using STRING (**Table S6**). All predicted BdAGOs were found to be either co-expressed or experimentally shown to interact with three proteins: Bradi1g36340.1, Bradi2g30160.1, and Bradi4g45065.1. BLASTP search of these protein sequences identified them as a 110-kDa U5 small nuclear ribonucleoprotein

TABLE 3 | Gene expression data as displayed on the *B. distachyon* eFP Browser (Winter et al., 2007; Sibout et al., 2017).

Gene ID	Assigned name	[1]Highest expression signal	[2]Peduncle, spikelet and stem nodes	[2]Root	[2]Leaf	[2]Seed
Bradi2g10360	BdAGO1	Whole_grain_11_DAF				
Bradi2g14147	BdAGO2	First_node_27_DAG				
Bradi2g10370	BdAGO3	First_node_10_DAG				
Bradi4g08587	BdAGO4	Whole_grain_2_years				
Bradi1g12431	BdAGO5	Not found in browser				
Bradi1g05162	BdAGO6	Endosperm_31_DAF				
Bradi1g28260	BdAGO7	Endosperm_11_DAF				
Bradi1g16060	BdAGO8	First_node_10_DAG				
Bradi1g36907	BdAGO9	First_node_10_DAG				
Bradi1g54977	BdAGO10	Whole_grain_11_DAF				
Bradi1g29577	BdAGO11	Upper_part_of_inclined_node_42_DAG				
Bradi5g18540	BdAGO12	Last_internode_35_DAG				
Bradi5g21810	BdAGO13	Last_node_35_DAG				
Bradi5g21800	BdAGO14	Last_internode_35_DAG				
Bradi3g51077	BdAGO15	Roots_10_DAG				
Bradi3g60697	BdAGO16	Roots_10_DAG				
Bradi1g77087	BdDCL1	Whole_grain_2_years				
Bradi1g15440	BdDCL2a	First_internode_27_DAG				
Bradi1g21030	BdDCL2b	Whole_grain_2_years				
Bradi3g29287	BdDCL3a	Lower_part_of_inclined_node_42_DAG				
Bradi2g23187	BdDCL3b	First_node_10_DAG				
Bradi5g15337	BdDCL4	Whole_grain_2_years				

[1]The tissue with the highest absolute expression level per gene ID is indicated (DAF, day after fertilization; DAG, day after germination).

[2]Summary of relative expression level per gene ID displayed for tissues; color indicates relative expression levels (log2, the control is calculated from all the samples displayed on the particular eFP browser view); dark blue (high for the transcript relative to control) to light blue (low for the transcript relative to control).

component CLO (Bradi1g36340.1), a putative GTP-binding/transcription factor (Bradi2g30160.1), and DNA-directed RNA polymerase V subunit 1 or DNA-directed RNA polymerase V subunit 1 (Bradi4g45065.1). In addition to the aforementioned proteins, BdAGO9 (classified in the AtAGO1/5/10 clade) was predicted to interact with seven other proteins, identified as three homeobox proteins knotted-1-like (Bradi1g12677.1, Bradi1g12690.1, Bradi1g57607.1), two GATA transcription factors (Bradi2g14890.1, Bradi2g45750.1), and two putative uncharacterized proteins (**Figure S2**).

DISCUSSION

In the present work, we investigated the phylogenetic relationships, domain, structure conservation, and predicted redundancy of AGO and DCL proteins in the model grass plant *B. distachyon*. Our findings imply that BdAGOs and BdDCLs have more copies and possibly greater diversification relative to *Arabidopsis*. One known example of such diversification in monocotyledonous plants is the rice AGO18, which confers antiviral immunity by sequestration of an miRNA (Wu et al., 2015). Since the presence of domains typical for AGO and DCL protein families serves as a selection criterion for proteins within this uninvestigated grass model species, we discuss phylogenetic relationships and predicted domain occurrence in detail.

Our analyses show that *Brachypodium*, like other grasses, contains one protein (BdDCL1) whose sequence groups with AtDCL1, one with AtDCL4 (BdDCL4), and two proteins each that group with AtDCL2 and AtDCL3 (Margis et al., 2006). Analysis of their predicted domain structures showed that BdDCL2b and BdDCL3b lack the dicer-dimer (DUF283) domain, known to mediate heterodimerization of AtDCL4 with its protein partners (Qin et al., 2010), but it is partially missing in two other DCLs (AtDCL3 and OsDCL2b, Margis et al., 2006). The second DSRM domain also was not predicted in either of the BdDCL2s. This finding is consistent with the previous discovery that AtDCL2 in *Arabidopsis* and OsDCL2a and OsDCL2b in rice also contain only one DSRM (Margis et al., 2006). This second DSRM domain has only a weak affinity for dsRNA, but it specifically binds to proteins of the HYPONASTIC LEAVES 1/dsRNA-binding protein family (Hiraguri et al., 2005; Margis et al., 2006). Since the DSRM domains mediate the transfer of the newly generated sRNA to the appropriate AGO protein (Parker et al., 2008), variations in the C-terminal architecture may influence which downstream partners and RNAi pathways are utilized by specific DCLs. The high level of divergence between DCL3a and DCL3b in several monocot species has led to the classification of DCL3b as a distinct type of DCL, termed DCL5. This monocot-specific class of DCLs has been retained for over 60 million years (Margis et al., 2006). It is is responsible for generating 24-nt-phased sRNAs in the male reproductive organs (Song et al., 2012). Interestingly, the predicted domain structure of BdDCL3b differs substantially from that of BdDCL3a, as it lacks both the DEXDc and HELICc domains (alongside the dicer-dimer domain) but contains an additional N-terminal DSRM (**Figure 4**, **Table S3**). Since the helicase domains are thought to mediate unwinding of the dsRNA (Zhang et al., 2004), the functionality of BdDCL3b is unclear. Mutations in AtDCL1 that impair helicase activity were previously shown to suppress miRNA accumulation (Liu

et al., 2012). However, comparable levels of transcripts for two splice variants of AtDCL2, one of which contains an altered helicase region, were detected throughout the *Arabidopsis* life cycle (Margis et al., 2006). Additional structural and biochemical analyses are therefore required to assess the role of BdDCL3b.

Phylogenetic analysis of the BdAGO protein family placed members in all three clades defined by *Arabidopsis* AGOs. Thus, the structural and functional differences of AtAGO proteins appear to be translated to the expanded *Brachypodium* family. For two of the three clades, the number of BdAGO and AtAGO family members was equivalent. By contrast, the AtAGO1/5/10 clade was highly expanded in *Brachypodium*, with four members (BdAGO9/11/12/15/16) grouped with AtAGO1. This member of the *Arabidopsis* family is associated with a range of functions, including processing of dsRNA from transgenes and exogenous sources, and RNAs involved in ckRNAi (Vaucheret et al., 2004; Weiberg et al., 2013). If the corresponding BdAGO members of this clade are found to have similar functions in PTGS-mediated transgene silencing, this information would be highly useful for developing RNAi-based protection strategies for cereal crops. AtAGO10 groups with the same BdAGOs, as expected considering the clade association with AtAGO1. AtAGO5, which is the third member of the AtAGO1/5/10 clade, groups with BdAGO5/6/7/10. By contrast, BdAGO1/2/3/4 were interspersed within the AtAGO4/6/8/9 clade, raising the possibility that these *Brachypodium* proteins are involved in TGS.

As displayed in our domain visualization (**Figure 3**), all 16 BdAGOs have a predicted PAZ domain. In AGOs, this domain recognizes the 3' end of the guide sRNA molecule, made accessible to the hydrophobic pocket of this nucleotide-binding domain by the typical 2'-O-methyl modification of the final sugar (Lingel et al., 2003; Cenik and Zamore, 2011). The MID domain recognizes the 5' nucleotide of the sRNA, thus giving preference of an AGO protein into which the sRNA will be loaded (Frank et al., 2012). In *Arabidopsis*, sRNA with a 5' U are sorted into AtAGO1, while AtAGO2 and AtAGO4 load sRNAs with a 5' A and AtAGO5 loads sRNAs with a 5' C (Mi et al., 2008). Our SMART/Pfam domain architecture search failed to identify a MID domain in any of the BdAGOs grouped in the AtAGO4/6/8/9 clade (BdAGO1/2/3/4) and with the AtAGO2/3 (BdAGO13/14) (**Table S2**), although this domain was reported in these proteins in a different study (Mirzaei et al., 2014). The specificity of sRNA sorting into particular AGOs can be further determined by the recognition of the sRNA secondary structure/base pairing by a QF-V motif present in the PIWI domain (Zhang et al., 2014). All *Arabidopsis* AGOs have the conserved QF-V motif, as do all 16 BdAGOs (**Figure 5**). The DEDD/H catalytic tetrad in the PIWI domain is also present in all but one of the BdAGOs. These active-site residues are critical for the RNase H-like endonuclease (slicer) activity exhibited by certain AGOs, which mediates sequence-specific cleavage of the target transcript. AtAGO1, AtAGO2, AtAGO4, AtAGO7, and AtAGO10 have been shown to have endonucleolytic activity toward target RNAs (Fang and Qi, 2016). Originally identified as a catalytic triad consisting of the residues DDH in most AtAGOs, but DDD in AtAGO2 and AtAGO3 (Höck and Meister, 2008), studies of yeast AGO revealed the importance of an invariant glutamate (E) residue, creating a catalytic tetrad (Nakanishi et al., 2012). This E residue is conserved in all *Arabidopsis* AGOs (Zhang et al., 2014). Consistent with these findings, MSA visualization of the BdAGO PIWI domains indicated that the majority of BdAGO proteins have the DEDH tetrad, except BdAGO13 and BdAGO14, which like their closest homologues AtAGO2 and AtAGO3, contain the DEDD tetrad (**Figure 5**). The only exception is BdAGO1, which is a short protein that terminates after 624 residues and lacks the last catalytic residue of the tetrad. Without the conserved catalytic residues, a specific AGO protein might induce gene silencing through means other than cutting, but Höck and Meister (2008) also discuss that the presence of a conserved catalytic triad does not mean the protein indeed has endonuclease activity. If an AGO does not display endonuclease activity, it may mediate PTGS *via* translation inhibition of the target RNA (Carthew and Sontheimer, 2009). Interestingly, the L1 and L2 linkers are predicted in all 16 BdAGOs as well (**Table S2**).

3D structure visualizations of all BdAGOs (**Figures 6**, **7** and **S1**) reinforce the conservation of the PAZ, PIWI, and MID domains (when predicted by SMART) and the catalytic tetrad residues in proximity within the PIWI domain (magenta spheres). The differences in the folding and looping linker regions within a certain group, relative to *Arabidopsis* AGOs, are shown in the model visualizations. Furthermore, the similarity between the 3D structures of BdAGOs that were predicted either to contain or lack a MID domain by the SMART/Pfam domain architecture search (e.g., **Figure 6**) reinforces the importance of comparing entire 3D models in order to gain insight into structure/function conservation. These structures are based on templates with better-known functional specificity and thus hint at the functions of the orthologs in Bd. As shown in **Table S4**, the templates used for modeling are based on either Argonaute 1 or Argonaute 2, with varying coverage and confidence values.

To assess the expression levels and locations of BdAGO and BdDCL family members, we analyzed the microarray-based expression data in the *B. distachyon* eFP browser (**Table 3**). Expression of *BdAGO* genes was observed in all four tissues assayed, although to varying extents. The expression patterns across the gene families indicate potential for functional redundancy. Notably, all members of the AtAGO1 clade (*BdAGO9/11/12/15/16*) show high and intermediate levels of expression in stem nodes and root tissue, respectively, while the *BdAGO1/2/3/4* proteins generally display high expression in stem nodes and seeds. Analysis of *BdDCL* gene expression revealed that most members of this family are highly expressed in stem nodes and/or seeds. *In vivo* experimental approaches are necessary to decipher whether the apparent co-expression of these genes indicates specific compartmentalization or complete/partial redundancy in the various RNAi processes, including environmental RNAi and ckRNAi pathways.

Finally, we used STRING to predict the interactome for members of the BdAGO family. This analysis indicates that all BdAGOs interact with three proteins (**Table S6**), as was expected because of the domain conservation within the family. In addition, several potential interactors were identified for BdAGO9, based on co-expression or experimental data (**Figure S2**, **Table S6**). Of these, DNA-directed RNA polymerase V subunit 1 was previously

shown to co-localize with, and possibly directly bind to, AtAGO4 *via* a so-called "Ago hook" (GW-rich domain), in order to facilitate the recruitment of AGO4 to chromatin to mediate TGS (El-Shami et al., 2007; Fang and Qi, 2016). Poulsen et al. (2013) have discussed that the binding of GW interactors to AGO make the loop with the E residue of the catalytic tetrad unavailable to the otherwise rigid DDD/H triad within the PIWI domain, thus offering an explanation of how the slicing activity is prevented in cases of silencing by translational inhibition. Moreover, GW containing proteins Needed for RDR2-independent DNA methylation and Silencing Defective 3 have been indicated in pathways bringing DNA/chromatin silencing together with RNAi proteins (Garcia et al., 2012; Pontier et al., 2012). Protein co-expression and interaction studies *in vivo* are necessary to confirm the identity and locations of these putative BdAGO interacting proteins. Due to the stringency of the prediction (excluding text mining data) and the lack of knowledge about the *Brachypodium* RNAi machinery, we were unable to predict additional interactions or to detect RNAi-related proteins that are known to interact with members of the AGO family in *Arabidopsis*. These include DCLs, HEN1 (involved in the methylation of sRNA 3' ends to prevent degradation), RDRs (RNA-dependent RNA polymerases that synthesize dsRNAs from single-stranded RNAs), and HSP90, the heat shock protein that binds to AtAGO1 and AtAGO4 to aid the loading of the sRNAs and RISC assembly (Fang and Qi, 2016; Nakanishi, 2016). Moreover, the predicted localization of the BdAGOs places the majority of them in the cytosol, except for BdAGO3 and BdAGO14, which are predicted to localize in the nucleus (**Table S7**). From what is known about *Arabidopsis* AGOs, AtAGO1 is proposed to have a localization in the nucleus and the cytoplasm (Vaucheret, 2008), while AtAGO4 localizes to the nuclear Cajal bodies (Höck and Meister, 2008).

In sum, based on *in silico* prediction, our data provide the first detailed functional insight into the AGO and DCL protein families in *Brachypodium*. In the context of plant–microbe interactions and ckRNAi, the *Brachypodium* orthologs of AtAGO1 are of special interest because microbial sRNAs are shown to be loaded onto AtAGO1 (Weiberg et al., 2013). Our predictions indicate a clade of BdAGOs structurally similar to AtAGO1, consisting of BdAGO9/11/12/15/16 (**Figure 7**). Elaborating on such similarities with the well-established clades of *Arabidopsis* AGOs and DCLs is a valuable basis for testing the hypothesis that BdAGO9/11/12/15/16 proteins are required for exogenous and endogenous dsRNA processing in HIGS, SIGS, and bidirectional ckRNAi in the grass model. Beyond what is predictable by *in silico* analysis, more data on expression patterns and interacting proteins are needed to further understand the role of these pillar proteins of RNAi pathways in cereals.

DATA AVAILABILITY STATEMENT

All datasets for this study are included in the article/ **Supplementary Files**.

AUTHOR CONTRIBUTIONS

EŠ, SZ, and KHK wrote the text. EŠ and SZ performed analysis and designed the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01332/full#supplementary-material

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REVIEW
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Management of Pest Insects and Plant Diseases by Non-Transformative RNAi

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Since the discovery of RNA interference (RNAi), scientists have made significant progress towards the development of this unique technology for crop protection. The RNAi mechanism works at the mRNA level by exploiting a sequence-dependent mode of action with high target specificity due to the design of complementary dsRNA molecules, allowing growers to target pests more precisely compared to conventional agrochemicals. The delivery of RNAi through transgenic plants is now a reality with some products currently in the market. Conversely, it is also expected that more RNA-based products reach the market as non-transformative alternatives. For instance, topically applied dsRNA/siRNA (SIGS – Spray Induced Gene Silencing) has attracted attention due to its feasibility and low cost compared to transgenic plants. Once on the leaf surface, dsRNAs can move directly to target pest cells (e.g., insects or pathogens) or can be taken up indirectly by plant cells to then be transferred into the pest cells. Water-soluble formulations containing pesticidal dsRNA provide alternatives, especially in some cases where plant transformation is not possible or takes years and cost millions to be developed (e.g., perennial crops). The ever-growing understanding of the RNAi mechanism and its limitations has allowed scientists to develop non-transgenic approaches such as trunk injection, soaking, and irrigation. While the technology has been considered promising for pest management, some issues such as RNAi efficiency, dsRNA degradation, environmental risk assessments, and resistance evolution still need to be addressed. Here, our main goal is to review some possible strategies for non-transgenic delivery systems, addressing important issues related to the use of this technology.

Keywords: RNAi, non-transgenic RNAi, RNA-based products, gene silencing, pest insects, plant diseases

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INTRODUCTION

From the earliest days of agriculture, mankind cultivated the land to feed their descendants, allowing for an increase in population growth over the years. Now, thousands of years later, modern agriculture is facing one of its biggest challenges: How are we going to produce food in a profitable, efficient, and sustainable way to feed about 10 billion people by 2050? Agricultural productivity has been facing several issues that limit crop production below its maximum potential, namely damage by insects, diseases, and competition with weeds. For instance, insects are responsible for 20 to 40%

of yield loss (Oerke, 2006). Moreover, researchers expect a 10 to 25% increase in insect damage per global temperature degree increment in the next years, with the main problems being in the temperate regions (Deutsch et al., 2018).

In an attempt to reduce the damage caused by pests, growers rely heavily on synthetic chemicals, which have been developed and applied since the 1930s. Pesticides allowed growers to increase production, improve product quality, and yield better profits. In 2012, growers around the world spent nearly $56 billion on pesticides, amounting to nearly 6 billion pounds of chemicals used in 2011 and 2012 (Atwood and Paisley-Jones, 2017). The high amount of chemicals used every year is leading to an increase in pesticide resistance, with a significant increase in resistance cases in insects (APRD 2019, https://www.pesticideresistance.org/search.php).

Modern agriculture is now entering the third green revolution, based on the significant progress in the use of reverse genetics to elucidate gene function and applying this knowledge in pest management. Major progress was made by Fire and Mello in 1998 by elucidating the gene-silencing mechanism in eukaryotic organisms named as RNA interference (RNAi) (Fire et al., 1998). RNAi, also known as Post Transcriptional Gene Silencing (PTGS), is a natural mechanism of gene regulation and is a defense system against viruses in eukaryotic cells (Hannon, 2002; Baum and Roberts, 2014) by degradation of the messenger RNA (mRNA) and reduction or complete elimination of the expression of a target gene (Fire et al., 1998).

Since the elucidation of the gene-silencing mechanism in eukaryotic organisms, significant advances have been made related to the use of this technique in the management of insect pest (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Huvenne and Smagghe, 2010; de Andrade and Hunter, 2016; Joga et al., 2016; San Miguel and Scott, 2016; Zotti et al., 2017) and plant diseases (Fu et al., 2005; Koch et al., 2013; Jahan et al., 2015; Koch et al., 2016; Wang et al., 2016b; Tiwari et al., 2017; Wang et al., 2017). Recently, the development by Bayer and approval of the SmartStax PRO maize carrying event MON87411 in Canada (2016) and the United States of America (USA) (2017) to control *Diabrotica virgifera virgifera* is considered a milestone in the use of RNAi technology in agriculture (Head et al., 2017). This technology is now available to growers as a tool for pest management. Delivery of double-stranded RNA (dsRNA) through this RNAi transformative approach (*i.e.,* transgenic plants) is a promising way to induce gene silencing in a specific pest (Baum and Roberts, 2014; Ghag, 2017), however it is not practical to every target organism or crop. Also, one of the key disadvantages of transgenic plants and seeds rely on regulatory approval, which takes years and is costly.

We are witnessing a constant decrease in the cost of dsRNA production together with an increased attraction from companies towards the development of improved dsRNA production techniques. It is therefore believed that non-transformative RNAi will soon reach the market (San Miguel and Scott, 2016; Cagliari et al., 2018; Mat Jalaluddin et al., 2018; Dubrovina and Kiselev, 2019). However, some issues are still hindering the development of non-transformative RNA-based products. In this paper, we aim to present the successful studies using non-transformative delivery systems and discuss limitations and possible solutions.

RNAi MECHANISM: FROM RNA DELIVERY TO GENE SILENCING

RNAi-based gene silencing can be triggered in the target organism by the supply of RNAs in two forms: (1) the delivery of dsRNA molecules or (2) the direct delivery of small RNAs (sRNAs). Currently, there are two major classes of sRNAs acting on the RNAi pathway: microRNAs (miRNAs) and small-interfering RNAs (siRNAs). MiRNAs are endogenously derived and involved in the regulation of gene expression, while siRNAs can be of exogenous origin from viruses or artificial supply (Preall and Sontheimer, 2005; Matranga and Zamore, 2007), or of endogenous origin from transposons (Lippman and Martienssen, 2004; Golden et al., 2008). It is known that, in most cases, insects take up dsRNAs longer than 50 bp but not sRNAs (Feinberg and Hunter, 2003; Saleh et al., 2006; Ivashuta et al., 2015), although some studies have shown that sRNA can trigger gene silencing (Borgio, 2010; Gong et al., 2013). By contrast, fungi and plants take up both dsRNAs and sRNAs (Koch et al., 2016; Wang et al., 2016b), suggesting that these organisms have a different uptake mechanism (Wang et al., 2017).

Once RNA molecules are delivered in the field (*i.e., via* transgenic plant, foliar spray, or trunk injection), they need to enter the cell of a target organism to trigger gene silencing. This process can occur through (a) direct or (b) indirect uptake (**Figure 1**). Direct uptake occurs when the RNA molecules are taken up through topical contact or feeding on plant tissues. By contrast, indirect uptake of RNA molecules involves first entering into the plant vascular system and then uptake by the insect/pathogen (Cagliari et al., 2018). The uptake process in the target pest is closely related to the delivery strategy, as demonstrated in several studies (**Table 1**).

Successful direct uptake *via* topical application has already been reported in different organisms (Pridgeon et al., 2008; El-Shesheny et al., 2013; Killiny et al., 2014). Zheng et al. (2019) reported that a dsRNA formulated in a nanocarrier plus a detergent was able to cross the cuticle in *Aphis glycines*, leading to a reduction of 95.4% in gene expression. Also, indirect uptake of dsRNA has been reported in some insects (Ghosh et al., 2017) and pathogens (Koch et al., 2016). However, there are some limitations related to the indirect uptake process, such as efficiency of translocation of the RNA molecules inside the plant vascular system and dsRNA processing by the plant RNAi machinery. Although it is known that RNAs can move through the plant vascular systems and plant cells (Melnyk et al., 2011; Molnar et al., 2011; Gogoi et al., 2017), some results have shown inefficient translocation of these molecules inside the plant vascular system. For example, in *Malus domestica* and *Vitis vinifera* treated with dsRNA and siRNA, the RNA molecules spread from treated to non-treated tissues but were restricted to the xylem vessels (Dalakouras et al., 2018). This study also found that in *Nicotiana benthamiana*, siRNA molecules were not efficiently translocated. In pathogens, studies on gene silencing

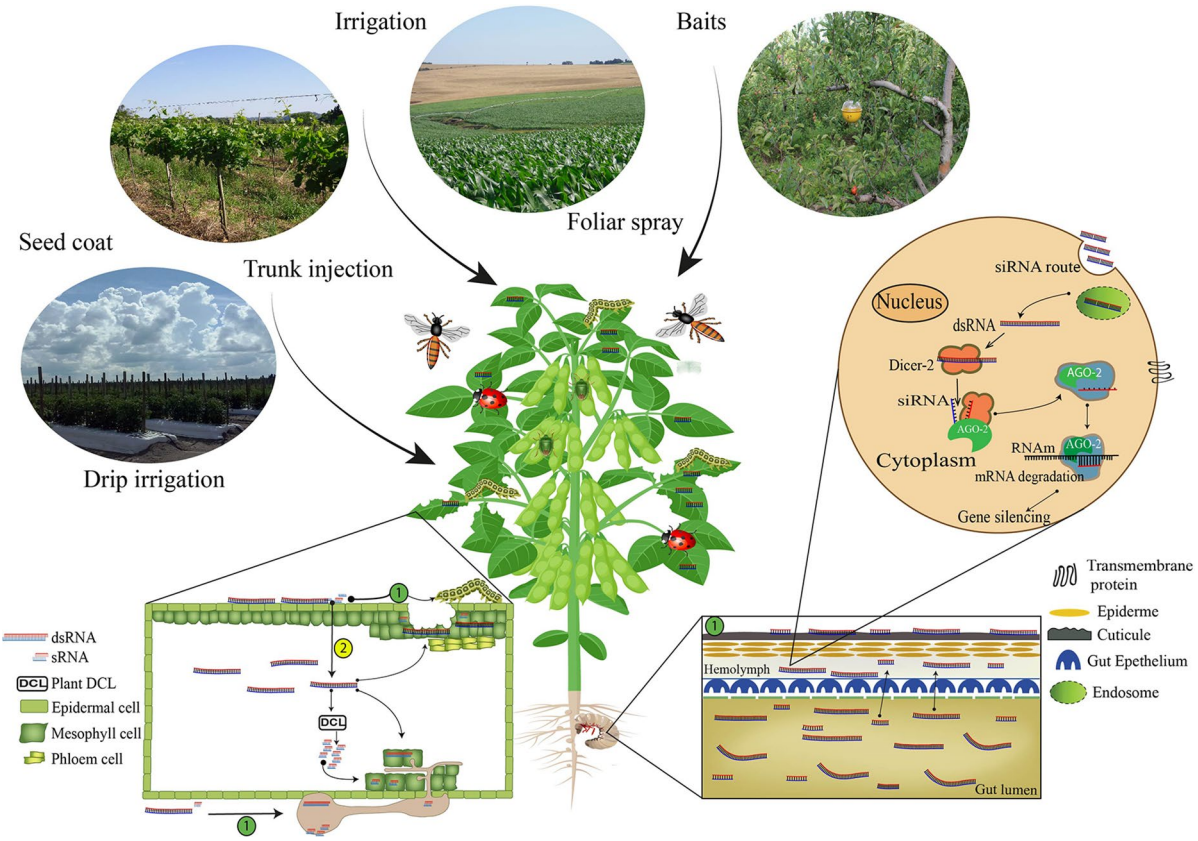

FIGURE 1 | Non-transformative delivery strategy routes for RNAi-based gene silencing induction. The first step to achieve successful RNAi-based gene silencing results *via* non-transformative approaches is the selection of the RNAs (dsRNA or siRNA) delivery strategy: Foliar spray, trunk injection, irrigation, drip irrigation, seed coat, baits, and powder or granules for soil applications. Once the RNAs are delivered, the insects and pathogens need to internalize the RNAs molecules, and this process can occur (1) directly or (2) indirectly. The direct uptake occurs when the organisms get in contact with the RNAs molecules during application or feed on tissues containing the RNA molecules on the surface. However, when the RNA molecules are absorbed, translocated in the plant vascular system and taken up by the organism (Koch et al., 2016), the process is classified as indirect uptake (Cagliari et al., 2018). Inside the organism system, the cell uptake of dsRNA can be mediated by transmembrane channel proteins such as sid-1 (Feinberg and Hunter, 2003; Aronstein et al., 2006; Kobayashi et al., 2012) or endocytosis (Saleh et al., 2006; Ulvila et al., 2006; Cappelle et al., 2016; Pinheiro et al., 2018; Vélez and Fishilevich, 2018). The RNAi-based gene silencing depends on the release at cellular levels of dsRNA or siRNA molecules (Carthew, 2009; Zotti and Smagghe, 2015). When dsRNAs are unloaded in the cytoplasm, these molecules are processed into siRNA fragments by an enzyme called Dicer 2 (DCR-2) (Meister and Tuschl, 2004; Tomari et al., 2007). The siRNA fragments are then incorporated into the RISC complex (RNA-induced Silencing Complex), which contains the Argonaute 2 (AGO-2) protein (Matranga et al., 2005; Miyoshi et al., 2005; Ketting, 2011), and, in a sequence-specific manner, bind to a complementary messenger RNA (mRNA), cleave it, prevent protein formation (Agrawal et al., 2003; Huvenne and Smagghe, 2010), and thus affect target organism survival.

TABLE 1 | Non-transformative delivery approaches and the relation between the organism location on the plant and the initial RNA uptake process.

Non-transformative delivery system	Insect/Pathogen location	RNA uptake process by the target organism	Reference
Soil drench; Drip irrigation; Irrigation	Roots; Stem; Leaves	Direct/Indirect	(Hunter et al., 2012; Li et al., 2015; Ghosh et al., 2017)
Seed coat or powder/granules	Roots; Stem	Direct/Indirect	–
Sprayable products	Stem; Leaves; Fruits/seeds	Direct/Indirect	(Hunter et al., 2012; Weiberg et al., 2013; de Andrade and Hunter, 2016; Wang et al., 2016b; Koch et al., 2016; San Miguel and Scott, 2016; Gogoi et al., 2017; Mitter et al., 2017b; McLoughlin et al., 2018; Niehl et al., 2018; Song et al., 2018; Gu et al., 2019; Worrall et al., 2019)
Trunk injection	Roots; Stem; Leaves; Fruits/seeds	Indirect	(Dalakouras et al., 2018; Hunter et al., 2012; Berger and Laurent, 2019)
Baits	Fruits	Direct	–

found evidence of external dsRNA processing into siRNAs (Koch et al., 2016; Konakalla et al., 2016; Mitter et al., 2017a). In *Hordeum vulgare*, dsRNA locally applied on detached leaves was taken up by plant cells, translocated through the vascular system, and processed into siRNAs by the plant Dicer enzyme, resulting in the inhibition of *Fusarium graminearum* growth in local and distal unsprayed leaves (Koch et al., 2016). In this study, the dsRNA molecules were found in xylem and phloem parenchymal cells, companion cells, mesophyll cells, and in trichomes and stomata, showing that the plant cells took up the dsRNAs. In citrus and grapevine plants treated with dsRNA, siRNAs were found in plants up to three months after treatment, indicating that the dsRNA was processed by the plant RNAi machinery (Hunter et al., 2012).

In some organisms, the process of dsRNA uptake by the cells can be mediated by transmembrane channel proteins such as sid-1 (Feinberg and Hunter, 2003; Aronstein et al., 2006; Kobayashi et al., 2012) or endocytosis (Saleh et al., 2006; Ulvila et al., 2006; Cappelle et al., 2016; Pinheiro et al., 2018; Vélez and Fishilevich, 2018). Recently, in *Drosophila*, scientists elucidated the involvement of nanotube-like structures, which mediate cell-to-cell trafficking of sRNA and RNAi machinery components, allowing gene silencing in cells and tissues distant from the uptake point (Karlikow et al., 2016). However, the uptake system of RNA varies among insects, even within the same order (Vélez and Fishilevich, 2018), resulting in variations in the efficiency of gene silencing.

Although a number of RNAi pathways use dsRNAs to generate sRNAs (*i.e.* microRNA and siRNA) (Bernstein et al., 2001; Ketting, 2011), in insects and fungi the siRNA pathway is known to be activated due to the presence of dsRNA molecules or a direct siRNA supply (Carthew, 2009; Zotti and Smagghe, 2015). Once inside the cell, dsRNAs are processed into siRNA fragments of ~20 base pairs (bp) in length by a ribonuclease III enzyme called Dicer 2 (DCR-2) (Meister and Tuschl, 2004; Tomari et al., 2007). The siRNA fragments are then incorporated into the RISC complex (RNA-induced Silencing Complex), which contains the Argonaute 2 (AGO-2) protein (Matranga et al., 2005; Miyoshi et al., 2005; Ketting, 2011). After unloading the non-incorporated passenger strand, the complex binds in a sequence-specific manner to the complementary mRNA, cleaving it, and preventing translation to protein (Agrawal et al., 2003; Huvenne and Smagghe, 2010).

The spread of the RNAi signal in the organism can be cell-autonomous or non-cell-autonomous (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). In cell-autonomous RNAi, silencing effects are observed only in the cells directly exposed to the dsRNA (Huvenne and Smagghe, 2010). By contrast, in non-cell-autonomous RNAi, the silencing effects are detected in exposed and non-exposed cells, even in different tissues (Whangbo and Hunter, 2008). Non-cell-autonomous RNAi is classified as environmental RNAi, a concept describing all processes in which dsRNA/siRNA are taken up from the environment by a tissue/cell and spread from one cell to another, or from one tissue type to another, through systemic RNAi (Huvenne and Smagghe, 2010). In plants, fungi, and the nematode *Caenorhabditis elegans*, the RNA-dependent RNA polymerase (RdRp) enzyme synthesizes secondary siRNAs by targeting single-stranded RNA molecules (ssRNA) and synthesizing a second strand, consequently generating dsRNA molecules and producing a systemic spread of the RNAi signaling (Zotti et al., 2017). The systemic nature of RNAi has already been observed in insects (Tomoyasu et al., 2008; Whyard et al., 2009; Wynant et al., 2012), however, the systemic RNAi mechanism is still unknown in this group. What is known about this process so far is that the dsRNA/siRNA spread from one cell to another cell or tissue is highly dependent on the cell's ability to take up the dsRNA or siRNA molecules (Vélez and Fishilevich, 2018), or on mediation through nanotube-like structures (Karlikow et al., 2016).

WHY USE NON-TRANSFORMATIVE DELIVERY STRATEGIES FOR PEST MANAGEMENT?

RNAi in crop protection can be achieved by plant-incorporated protectants (PIPs) through plant transformation (*i.e.,* transgenic plants) or by non-transformative strategies through a spray-induced gene silencing (SIGS) process (**Table 2**). Regardless of the delivery strategy, the use of RNA-based products to confer plant protection against insects and pathogens is a potential alternative to conventional pesticides (Koch et al., 2016).

Currently, approved RNAi-based GM plants are based on ncRNA (non-coding RNA) to control insects (8%) and diseases (27%) or to improve specific plant traits (65%), with an increase in approved events over the last years (**Figure 2**). In 2016, the first transgenic RNAi crop (SmartStax PRO maize) combining *Bt* (*Bacillus thuringiensis*) toxin with RNAi for insect control was released for cultivation in Canada and a year later in the USA (Head et al., 2017). In general, the delivery of dsRNA in the field is facilitated by the use of GM plants, however, this strategy still cannot be adopted in all plants/crops due to the

TABLE 2 | Different features affecting the development of RNAi-based products: Transformative vs. Non-transformative methods.

Feature	Strategy	
	Transformative	**Non-transformative[1]**
Development time	High	Low[2]
Development costs	High	Low
Feasibility according to culture	Unviable for some plant species	Viable for all cultures[1]
Delivery of sRNA	Continuous	Transient
Feasibility according to the pest	Most pests can be targeted due to continuous dsRNA supply feature	Not all pests can be targeted due to recalcitrant features
Development of resistance	High	Low
Regulatory process	Extensive	Simple
Acceptance by consumers	Low	High

[1]Non-transformative delivery approaches: foliar application, trunk injection, and irrigation water among others; [2]Non-transforative strategy compared to transformative strategy.

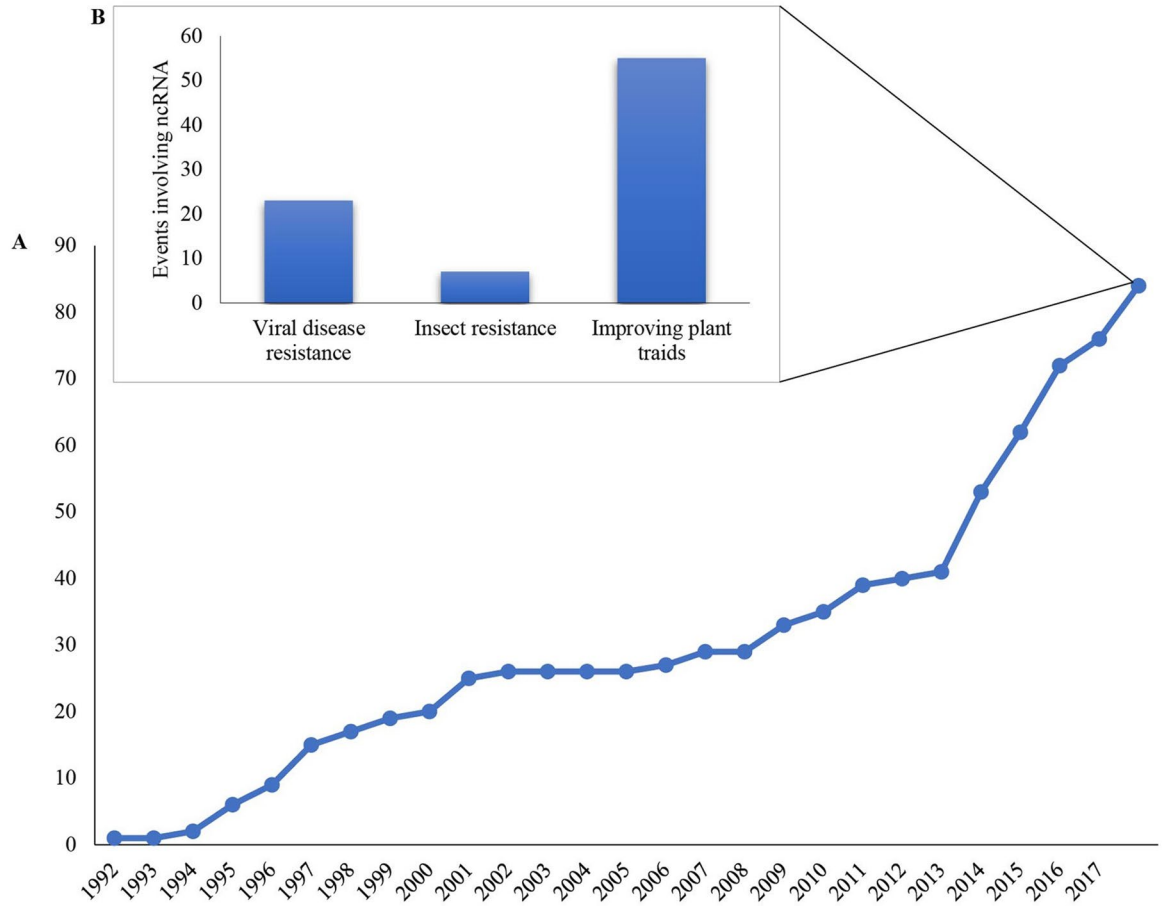

FIGURE 2 | Accumulated, approved genetically modified events based on non-coding RNA (ncRNA) worldwide for cultivation since 1992. **(A)** Total approved ncRNA GM events worldwide since the first ncRNA approved event in 1992; **(B)** Number of ncRNA GM events according to the desired features. The data used to make the graphics were compiled from the GM Approval Database at the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) (http://www.isaaa.org/gmapprovaldatabase/default.asp).

high cost of production and the long time for development. For instance, the commercial availability of "HoneySweet," a cultivar resistant to the *Plum pox virus* (PPV), took 20 years to reach the market (Scorza et al., 2013). Also, there are no established transformation protocols for most of the cultivated plants, which may cause a substantial delay in the development of RNAi-based GM plants (Mitter et al., 2017b). Therefore, alternative strategies for the delivery of RNA biopesticides are necessary and could provide alternative ways to use this technology in the field. Given that non-transgenic RNAi-based products would silence genes without introducing hereditary changes in the genome, it is expected that they will not be regulated as GM products, thereby reducing the time and processes for their release to use as well as potentially improving public acceptance (Cagliari et al., 2018).

Studies are being carried out prospecting non-transformative approaches to control insects, diseases, nematodes, and weeds, and it is expected that RNAi-based products will reach the market in the form of sprayable products for foliar application, trunk injection, root dipping, or seed treatment as direct control agents (Zotti and Smagghe, 2015; San Miguel and Scott, 2016; Zotti et al., 2017; Cagliari et al., 2018; Berger and Laurent, 2019; Dubrovina and Kiselev, 2019). The RNA-based new generation of biopesticides could circumvent the technical limitation of plant transformation and the public's concerns about GM plants, providing an easy-to-use tool for crop production and storage, as well as an environmentally friendly pest management strategy (Wang et al., 2017; Zotti et al., 2017). Furthermore, RNA-based biopesticides could be efficiently designed to target multiple insects or pathogen species.

The development of resistance is an important point regarding the use of non-transformative delivery strategies. Although dsRNAs longer than 200 nucleotides result in many siRNAs post-cleavage, maximizing the RNAi response and reducing resistance issues (de Andrade and Hunter, 2016), in transgenic plants there is a continuous supply of dsRNA, which increases the selection pressure and favors resistance development in the population. The development of RNAi resistance may be related to a reduction in cellular uptake (Khajuria et al., 2018), mutations in mRNA, production of RNAi suppressors (Zheng et al., 2005), upregulation of the target gene or downregulation of the silencing

machinery genes (Garbutt and Reynolds, 2012), increased nuclease activity (dsRNases) (Spit et al., 2017), or even behavioral changes. However, when non-transformative delivery techniques are adopted, insects and pathogens have limited exposure to the dsRNA molecules due to the transient feature of such molecules, preventing the development of resistance in the target organisms.

Non-transformative delivery methods can be developed for use on several crops, targeting pests in different regions. Although GM event approval is more complicated, RNA-based non-transformative products will also undergo regulation procedures, although they will probably be less complicated and time-consuming than for GM plants. Also, an important aspect related to the legislation of non-transformative products is that RNA-based biopesticides will probably need to be approved in only the producing country, unlike GM plants, which needs approval in both import and export countries.

SUCCESSFUL NON-TRANSFORMATIVE DELIVERY CASES

Based on the advances made in the last decades regarding the use of RNAi in crop protection, it is believed that this technology will soon reach growers as dsRNA/siRNA-based products (Cagliari et al., 2018; Mat Jalaluddin et al., 2018). The application of RNAs targeting essential insect or fungi genes can significantly impair growth, increase mortality rate, and, in some cases, suppress insecticide/fungicide resistance (Pridgeon et al., 2008; Killiny et al., 2014). Although RNAi is not currently functional in every delivery method and every insect life stage or target gene (San Miguel and Scott, 2016), this technology has great potential, especially for insects and diseases with high insecticide- and fungicide-resistance problems.

On the development of non-transformative delivery technologies, in 2011 the Monsanto company published the patent WO 2011/112570 in which the company uses sprayable polynucleotide molecules to regulate gene expression in plants (Sammons et al., 2011). According to the patent, dsRNAs, siRNAs, and even single-stranded DNA oligonucleotides triggered efficient local and systemic silencing of *N. benthamiana* endogenous genes. However, in another experiment, researchers were unsuccessful in inducing gene silencing in plants through siRNA application, including spraying, syringe injection, or siRNAs infiltration, yet they achieved success through high-pressure spraying of siRNAs (Dalakouras et al., 2016).

The delivery system varies according to the target organism and crop (**Table 1**). The selection of the delivery strategies (*i.e.,* foliar sprays, irrigation, trunk injection, and baits among others) is the first step to achieve good control results, determining the success of the technology usage. The correct choice of delivery system will expedite the entire process and save years of development and commercialization (de Andrade and Hunter, 2016). Hence, the main non-transformative delivery methods and their applications in insect and disease management, shown in **Table 3**, will be discussed further in the following sections.

Foliar Application

For pests feeding/growing on stems, foliage, or fruit/seeds, foliar spraying may be an alternative for the delivery of RNA molecules. Thus, the RNA-based formulations are evaluated similarly to topical insecticides where the RNA solution is sprayed on leaves, fed to the target insects, and the effects are observed (de Andrade and Hunter, 2016). Due to the chemical properties of RNAs, a short half-life is expected compared to chemical pesticides. Sprayable RNAs would therefore be an environmentally friendly alternative to synthetic pesticides (Fire and Won, 2013; Wang and Jin, 2017).

One of the first studies exploring the applications of sprayable RNA molecules to control insect pests was conducted using siRNA molecules against the diamondback moth, *Plutella xylostella.* Mortality rates of ~60% were observed when larvae were fed with *Brassica* spp. leaves sprayed with chemically synthesized siRNAs targeting the *acetylcholine esterase* genes *AchE2* (Gong et al., 2013). In an attempt to control the Colorado potato beetle, *Leptinotarsa decemlineata,* foliar application of naked dsRNA targeting the *actin* gene was sufficiently stable for at least 28 days under greenhouse conditions, resulting in significant insect control (San Miguel and Scott, 2016). The same strategy was tested with the aim to control the xylem-feeding leafhopper (*Homalodisca vitripennis*), the phloem-feeding Asian citrus psyllid (*Diaphorina citri*) (Hunter et al., 2012), and the Diaprepes root weevil (*Diaprepes abbreviates)* on citrus leaves, showing a promising alternative to control these insects (de Andrade and Hunter, 2016). In tomato leaves gently rubbed with dsRNA solution, the molecules were rapidly absorbed by tomato plants and were taken up by aphids (*Myzus persicae*), mites (*Tetranychus urticae*), and in fewer numbers, whiteflies (*Trialeurodes vaporariorum*) (Gogoi et al., 2017). Hence, siRNA molecules were only detected in tomato plants, aphids and mites, and they were absent in the whiteflies, in which the dsRNA amounts did not reach the threshold necessary to induce RNAi machinery.

The use of RNAs in foliar application to manage pathogen infection and resistance in crops was also explored. In 2013, scientist discovered that Dicer-like protein 1 and 2 from *Botrytis* (Bc-DCL1; Bc-DCL2) fungus produces small RNAs (Bc-sRNAs), which are delivered into plant cells, silencing host immunity genes (Weiberg et al., 2013). Years later, researches applied siRNAs and dsRNAs targeting *Botrytis cinerea* DCL1 and DCL 2 (Bc-DCL1/2) onto the surface of fruits (tomato, strawberry, and grape), vegetables (lettuce and onion), and flowers (roses), which resulted in the significant inhibition of grey mold disease development (Wang et al., 2016b). In both cases, naked dsRNA/siRNA treatment was able to protect plants from the microbial pathogen for up to ten days after spraying. Moreover, these researchers showed that plants infected with another pathogen, *Verticillium dahlia,* displayed severe wilt disease symptoms, indicating that Bc-DCL1/2 RNAs were specific to *B. cinerea* DCL genes and did not cause non-target effects (Wang et al., 2016b). In the same year, a breakthrough work showed the foliar application of dsRNA targeting the *cytochrome P450* (*CYP3*) gene in *F. graminearum,* resulting in the successful inhibition of fungal growth in local directly sprayed leaves as well as the distal

TABLE 3 | Non-transformative delivery strategies for insects, pathogens, and virus management.

Target pest	Crop	Delivery strategy	Target gene	Molecule	Size	Molecule concentration	Results	Reference
				Insects				
Plutella xylostella	Kale	Foliar spray	AChE2	siRNA	18–27 bp	200 µg/ml	Approximately 60% mortality.	(Gong et al., 2013)
Leptinotarsa decemlineata	Potato	Foliar spray	Actin	dsRNA	50 – 297 bp	5 µg leaf $^{-1}$	Significant mortality in dsRNA length-depend pattern.	(San Miguel and Scott, 2016)
Diaprepes abbreviates	Citrus	Foliar spray	Not informed	dsRNA	Not informed	Not informed	Control started 4-5 days after dsRNA application.	(de Andrade and Hunter, 2016)
Diaphorina citri; Bactericera cockerelli; Homalodisca vitripennis	Citrus approximately 2.5 m tall and Grapevines	Trunk injection; root drench	Arginine kinase	dsRNA	Not informed	2 g in 15 liters of water	Insects successfully uptake dsRNA from the treated plants; dsRNA was detected in plants for at least 57 days.	(Hunter et al., 2012)
Nilaparvata lugens	Rice	Roots soaking	Ces CYP18A1	dsRNA	Not informed	1 mL (1.0 mg mL^{-1} of water)	Gene knocked down; nymph mortality.	(Li et al., 2015)
Ostrinia furnacalis	Maize	Irrigation	KTI	dsRNA		10 mL (0.5 mg mL^{-1} water)	Gene knocked down; larval mortality.	
Myzus persicae *Tetranychus urticae* *Trialeurodes vaporariorum*	Tomato	Foliar application	ZYMV HC-Pro	dsRNA	588 bp	10.5 µg dsRNA in 10 µL water	Insect successfully uptake dsRNA; the dsRNA was processed into siRNA by the insect RNAi machinery. Low dsRNA uptake; No siRNA in insects.	(Gogoi et al., 2017)
Halyomorpha halys	Green beans	Soaking	JHAMT Vg	dsRNA	200-500 bp	300 µl (0.017 µg µL^{-1} of water) 300 µl (0.067 µg µL^{-1} of water)	Significant reduction in gene expression.	(Ghosh et al., 2017)
Planococcus citri	Tobacco	VIGS using recombinant TMV	Actin CHS1 V-ATPase	siRNA	Not informed	–	Crawlers feed on recombinant TMV-infected plants showed lower fecundity and pronounced death.	(Khan et al., 2013)
Bactericera cockerelli	Tomato Tomatillo Tobacco	VIGS using recombinant TMV	Actin	siRNA	21 nt	–	Gene knocked down in insects feed on these plants; Insects fed on infected tomatillo plants showed a decreased progeny production.	(Wuriyanghan and Falk, 2013)
Diaphorina citri	Citrus	VIGS using recombinant CTV	Awd	siRNA	20-22 nt	–	Adults showed malformed-wing phenotype and increased mortality.	(Hajeri et al., 2014)
Phenacoccus solenopsis	Tobacco	VIGS using recombinant PVX	Bur V-ATPase	siRNA	–	–	Insects fed on treated plants showed physical deformities or died.	(Khan et al., 2018)
Drosophila melanogaster	–	VIGS using recombinant FHV; microinjection	RPS13 Vha26 Alpha COP	siRNA	–	–	Significantly higher mortality in insects.	(Taning et al., 2018)
Helicoverpa armigera	–	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115*;* artificial diet coated with engineered bacteria	AK	dsRNA	379-426 bp	30 µL (10^9 cells)	Knocked down the target gene caused drastic reductions in body weight, body length, and pupation rate, resulting in high mortality.	(Ai et al., 2018)

(Continued)

TABLE 3 | Continued

Target pest	Crop	Delivery strategy	Target gene		Molecule	Size	Molecule concentration	Results	Reference
Spodoptera exigua	Chinese cabbage	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115	INT		dsRNA	410 bp	10^7 cells per larva	Significant reduction of the SeINT expression resulting in insect mortality; Pretreatment with an ultra-sonication increased the insecticidal activity of the recombinant bacteria, and treated larvae became s susceptible to Cry toxin.	(Kim et al., 2015)
	–	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115*;* artificial diet containing engineered bacteria	CHSA		dsRNA	635 bp	High dose (250X), medium dose (50X), and low dose (10X) based on the dilution factors.	Significant reduction in survival rates. Levels of target gene expression, tissue structure, and survival rates were dose-dependent.	(Tian et al., 2009)
Lymantria dispar	–	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115*;* diet with engineered bacteria	Locus 365 Locus 28365		dsRNA	–	300 µl of bacteria culture	Target-gene knocked down, reduction in body mass and egg masses.	(Ghosh and Gundersen-Rindal, 2017)
Mythimna separata	–	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115*;* artificial diet containing engineered bacteria	Chi		dsRNA	700 bp	–	Target gene knocked down after oral delivery of engineered bacteria, resulting in resulted in increased mortality and reduction in body weight of the feeding larvae.w	(Ganbaatar et al., 2017)
Bactrocera dorsalis	–	dsRNA expressed in bacteria, using recombinant *E. coli* strain HT115*;* artificial diet containing engineered bacteria	Rpl19 V-ATPase Rab11 Noa		dsRNA	–	200 ml 250X of bacteria culture expressing dsRNA.	Successful gene silencing of the target genes after insects were fed on a diet containing engineered bacteria. An over-expression of the target genes after continuously supply of engineered bacteria was also observed.	(Li et al., 2011)
Bemisia tabaci	Hibiscus	dsRNA expressed in fungus, using engineered *Isaria fumosorosea*	TLR7		dsRNA	548 bp	$2x10^7$,$1x10^7$,$5×10^6$, $2.5x10^6$ spores mL^{-1}	The engineered IfB01-TRL7 strain increased the mortality of whitefly nymphs compared to the IfB01 strain. The IfB01-TRL7 strain also show higher virulence, with decreased and shortened values of LC50 and LT50.	(Chen et al., 2015)
Manduca sexta	Tobacco	VIGS using recombinant TRV	DCL1 DCL2 DCL3 DCL4 CYP6	In tobacco plants In tobacco hornworm	dsRNA	≥ 300 bp	–	Knocked down of the DCL target genes in engineered tobacco plants to express a 312 bp fragment of *Ms*CYP6B46 gene increased the gene silencing results.	(Kumar et al., 2012)
					Diseases				
Fusarium graminearum	Barley	Foliar spray	CYP3		dsRNA	791 bp	500 µL (20 ng $µL^{-1}$ of water)	Inhibition of fungal growth.	(Koch et al., 2016)
SCMV	Maize	Bacterial crude extract foliar spraying (*E. coli* strain HT115)	CP		dsRNA	147-247 bp	One-half diluted extraction crude	Inhibition of SCMV infection.	(Gan et al., 2010)

(Continued)

TABLE 3 | Continued

Target pest	Crop	Delivery strategy	Target gene	Molecule	Size	Molecule concentration	Results	Reference
Botrytis cinerea	Tomato, Strawberry, Grape, Lettuce, Onion, Rose	Foliar application	DCL1 DCL2	sRNA dsRNA sRNA dsRNA	21-24 nt 252 bp 21-14 nt 238 bp	400 µl (20 ng µL^{-1})	Both sRNA and dsRNA were uptake by the fungus resulting in fungal growth inhibition.	(Wang et al., 2016b)
Sclerotinia sclerotiorum *Botrytis cinerea*	Canola	Foliar spray	59 target genes	dsRNA	200-450 bp	10–25 µL of 200–500 ng dsRNA plus 0.02–0.03% Silwet L-77.	From the 59 dsRNAs tested, 20 showed antifungal activity with a reduction in lesion size ranging from 26–85%.	(McLoughlin et al., 2018)
BCMV	Tobacco; cowpea	Foliar spray	Nib CP	dsRNA naked or loaded onto LDH	480 bp 461 bp	100 µg of in a 1 mL or 250 ng of dsRNA.	Plants were protected from aphid-mediated virus transmission.	(Worrall et al., 2019)
Fusarium asiaticum	Wheat	Foliar spray	Myosin 5	dsRNA	496 bp	0.1 pM	Reduced pathogen sensitivity to phenamacril with a reduction in infection.	(Song et al., 2018)
PPV	Tobacco	Bacterial crude extract foliar spraying (*E. coli* strain HT115)	IR 54	hpRNA	977 bp	Dilution series (1/2 to 1/20) using 3 µg of total nucleic acid/µl.	Dilutions of 1/10 or less did not display disease symptoms upon completion of their life cycles	(Tenllado et al., 2003)
PMMoV			HC; CP	dsRNA	1492 bp; 1081 bp	One-half diluted French Press preparations derived from engineered bacteria.	Plants treated with dsRNA-expressing preparations showed no virus symptoms (HC: 82% or CP: 73%).	
TMV	Tobacco	Bacterial crude extract foliar spraying (Different *E. coli* strain tested)	CP	dsRNA	480 bp	One-half diluted French Press preparations derived from engineered bacteria.	M-JM109 or M-JM109lacY strains and the pGEM-CP480 vector exhibited the best results producing great quantities of dsRNA. Tobacco plants sprayed with dsRNA crude bacterial extract showed inhibition in TMV infection.	(Yin et al., 2009)
PMMoV CMV	Tobacco Cowpea	Foliar spray	RP 2b supressor	dsRNA naked or loaded onto LDH	977 bp 330 bp	125 µL per cm^2 (1.25 µg of dsRNA and/or 3.75 µg of LDH).	Virus protection for at least 20 days.	(Mitter et al., 2017a)
Fusarium asiaticum. *Botrytis cinerea* *Magnaporthe oryzae* *Colletotrichum truncatum*	Wheat Cucumber Barley Soybean	Foliar spray after leaves were wounded using quartz sand	β2-tubulinX	dsRNA	480 bp	40 ng µL^{-1} of water	Antifungal activity against these fungi with a reduction in the dosage of carbendazim fungicides necessary to control the pathogens.	(Gu et al., 2019)

AChE2, acetylcholine esterase; CP, Coat Protein; Ces, carboxylesterase; ZYMV, Zucchini yellow mosaic virus; JHAMT, Juvenile hormone acid O-methyltransferase; Vg, Vitellogenin; CYP: cytochrome P450; KT, Kunitz-type trypsin inhibitor; DCL, Dicer-like; BCMV: Bean common mosaic virus; PMMoV, Pepper mild mottle virus; CMV, Cucumber mosaic virus; LDH, double-layered hydroxide; RP, Replicase; CTV, Citrus tristeza virus; Awd, abnormal wing disc; BUR, Bursicon; FHV, Flock house virus; RPS13, Ribosomal protein S13; Vha26, Vacuolar H[+]-ATPase 26kD E subunit; Alpha COP, Alpha-coatomer protein; AK, Arginine kinase; INT, β1 integrin gene; CHSA, Chitin synthase gene A; Chi, chitinase; Rpl19, ribosomal protein Rpl19; Sec23, Protein transport protein sec23; vATPaseE, Vacuolar ATP synthase subunit E; vATPaseB, Vacuolar ATP synthase subunit B; COPβ, Coatomer subunit beta; SCMV, Sugarcane Mosaic Virus; HC, Helper component; IR, replicase; TLR7, Toll-like receptor 7; LC50, Lethal Concentration 50; LT50, Lethal Time 50; VIGS, Virus-induced gene silencing.

non-sprayed leaves in barley plants (Koch et al., 2016). DsRNA foliar applications also conferred protection against *Sclerotinia sclerotiorum* and *B. cinerea* in *Brassica napus* (McLoughlin et al., 2018). Due to the relative ease of design and the high specificity and applicability to a wide range of pathogens, the use of "RNA fungicides" as anti-fungal agents offers unprecedented potential as a new plant protection strategy that is also less harmful to the environment.

Furthermore, the use of RNA to target pathogen resistance to regular fungicides is also under development. Spraying wheat plants with dsRNA targeting the *Fusarium asiaticum myosin 5* gene resulted in increased pathogen sensitivity to phenamacril with a reduction in infection (Song et al., 2018). Although dsRNA has a high specificity, it is also possible for dsRNA molecules to target a specific group. DsRNA molecules of a *β2-tubulin* gene derived from *F. asiaticum* suppressed the fungal activity of *F. asiaticum*, *B. cinerea*, *Magnaporthe oryzae*, and *Colletotrichum truncatum* in wheat, cucumber, barley, and soybean, respectively (Gu et al., 2019). Alongside this, the dsRNA molecule also functioned to reduce the dosage of carbendazim (MBC) fungicide to control the pathogens. Thus, the combination of dsRNA and site-specific fungicide can be a control strategy against resistant pathogen infection in the field, rather than the individual use of dsRNA or fungicides.

Co-inoculation of synthesized dsRNA to protect plants against a virus/viroid is effective at preventing virus infection in a range of plants through mechanical inoculation, thereby increasing the prospect for foliar dsRNA application in virus management in plants (Tenllado and Díaz-Ruíz, 2001; Carbonell et al., 2008; Šafářová et al., 2014; Konakalla et al., 2016). Recently, Niehl et al. (2018) suggested the term "plants vaccines," citing the use of sprayable dsRNA to control the *Tobacco mosaic virus* (TMV) in tobacco, similarly to vaccines for animals that use dead or living (but weakened) microorganisms. These researchers used fragments of the virus' genetic material to produce the "vaccines" (dsRNA) together with the plant's immune system as a defense mechanism. This system opens a range of opportunities for the use of RNAi in a non-transformative approach in the control of viruses in crops.

The potential applications of SIGS for plant protection have had significant improvement due to the recent advances in nanoparticle technology. To overcome problems related to dsRNA stability, a double-layered hydroxide (LDH) nanoparticle was developed and combined with dsRNA molecules to yield "BioClay" (Mitter et al., 2017b). The clay nanoparticles are positively charged and thus bind and protect the negatively charged dsRNAs; delivery occurs when atmospheric carbon dioxide and moisture reacts with the clay nanoparticles, breaking the LDH and gradually releasing the dsRNAs. Using the dsRNA-LDH complex, researchers were able to achieve long-term gene silencing results by protecting tobacco plants from a virus for up to 20 days with a single spray, extending the period from five to seven days using naked dsRNA (Mitter et al., 2017a; Mitter et al., 2017b). In another experiment, researchers sprayed tobacco and cowpea plants with BioClay nanosheets of dsRNA from the coat protein from the *Bean common mosaic virus* (BCMV) five days before exposure to viruliferous aphids (Worrall et al., 2019). The researchers found that BioClay molecules protected plants from BCMV infection due to aphid-mediated virus transmission and considered this an important step toward the development of a practical application of dsRNA in crop protection. These results using sprayable dsRNA are encouraging, and although more progress is needed on several fronts, RNA-based biopesticides are expected to reach the market soon. Monsanto is developing the use of RNAi through a technology called "BioDirect," in which dsRNA formulation is applied exogenously to protected plants against insect and pathogen attack (https://monsanto.com/innovations/agricultural-biologicals/). Syngenta scientists are also developing lines of biocontrol products based on RNAi to protect potato plants from the attack of the Colorado potato beetle (https://www.youtube.com/embed/BiVZbAy4NHw?ecver=1). These technologies will help growers to improve pest control in crops, resulting in increased yields and improved quality.

Trunk-Injection

The use of trunk injection to deliver dsRNA to control insects has been tested and showed great progress, especially in perennial plants such as citrus. Developed citrus plants (2.5 meters tall) and grapevines were treated with 2 g of dsRNA in 15 L of water solution applied by root drench and injection into the trunk, and dsRNA was taken up into whole plant systems over three months (Hunter et al., 2012). In citrus plants, the dsRNA was detected in the psyllid and the spittlebug from five to eight days after entering the plants, allowing the development of pest suppression.

Recently, researchers showed that hairpin RNAs (hpRNAs) and siRNAs delivered through petiole absorption or trunk injection to *M. domestica* and *V. vinifera* plants were restricted to the xylem vessels and apoplast, being efficiently translocated (Dalakouras et al., 2018). Due to this characteristic, the plant Dicer-like (DCL) endonucleases were unable to process the hpRNA. Injected RNA molecules were thus detected in plants for at least ten days post-application. However, when siRNA was delivered to *N. benthamiana* through petiole absorption, the molecules were not efficiently translocated. These innovative methods may have a significant impact on pest management against chewing or xylem sap-feeding insects and eukaryotic pathogens that reside in the xylem, allowing an essay reposition of the RNA-based solution and efficient plant protection for a longer period.

Irrigation

Hunter and collaborators showed that the dsRNA applied through a root drench in adult citrus plants (2.5 m tall) could effectively control psyllids and leafhoppers for up to 57 days (Hunter et al., 2012). They were able to detect the RNA molecules in the citrus plants for over three months. Rice plant roots soaked in a solution containing dsRNA targeting *carboxylesterase* (*Ces*) and *CYP18A1* genes from the brown planthopper (BPH), *Nilaparvata lugens*, significantly knocked down these genes, resulting in high mortality when BPH nymphs were fed on treated plants (Li et al., 2015). This study also showed maize seedlings irrigated with dsRNA of the Kunitz-type trypsin inhibitors (dsKTI) from the Asian corn borer (ACB), *Ostrinia furnacalis,* and this resulted

in high larval mortality rates. Recently, Ghosh and collaborators showed that *Halyomorpha hayls* nymphs fed on green beans soaked in dsRNA solution targeting *JHAMT* (*Juvenile hormone acid O-methyltransferas*) and *Vg* (*Vitellogenin*) genes resulted in a significant reduction in gene expression, indicating that RNAi can be efficiently employed through vegetable delivery in plant-sap-feeding insects (Ghosh et al., 2017). The delivery of gene silencing molecules through irrigation can be an alternative for crops that use irrigation in the normal growing system, allowing for the continuous supply of RNA molecules. However, Dubelman et al. (2014) reported short persistence of dsRNA molecules in the soil, with a rapid breakdown within 2–3 days. Therefore, the dsRNA stability in the soil is still an issue affecting RNAi efficiency (Joga et al., 2016), and the feasibility of this delivery strategy relies on the advances of formulations to protect RNA molecules from degradation.

Microbe-Induced Gene Silencing

Many microbes such as viruses, bacteria, yeasts, and fungi can be engineered to generate a vector for RNAi induction through the continuous production of dsRNA into the host, and this is being considered as a promising dsRNA delivery method for insect and disease management (Fjose et al., 2001; Whitten et al., 2016; Cagliari et al., 2018; Dubrovina and Kiselev, 2019; Goulin et al., 2019).

Virus-induced gene silencing (VIGS) is a naturally occurring and very effective defense system that is consistent with the normal dynamics of host–pathogen interactions and which is widely harnessed as a powerful tool for the study of gene function in plants (Ratcliff et al., 1997; Waterhouse et al., 2001; Lu et al., 2003; Robertson, 2004; Baulcombe, 2015). VIGS is transiently transformative and does not cause alterations in the plant's genetic composition, unlike stable RNAi and mutant plants. Furthermore, VIGS can be transmitted to plant progeny and actively co-opts the plant for expression of dsRNA (Senthil-Kumar and Mysore, 2011). Moreover, VIGS enables high throughput screening of potential targets genes to control insect pest (Gu and Knipple, 2013; Nandety et al., 2015; Kolliopoulou et al., 2017). In Lepidoptera, three midgut-expressed *CYP* genes in *Manduca sexta* were targeted through the engineering of *Tobacco Rattle Virus* (TRV) for dsRNA delivery in *Nicotiana attenuata* (Kumar et al., 2012). Also, plant-virus based dsRNA delivery vectors are promising tools for targeting phloem-feeding insects because almost all plant-infecting viruses infect and move systemically *via* the phloem (Nandety et al., 2015). To demonstrate this, researchers used a recombinant TMV to express RNAi effectors in *N. benthamiana* plants against the citrus mealybug (*Planococcus citri*) and observed lower fecundity and a pronounced death of crawlers after feeding on recombinant TMV-infected plants (Khan et al., 2013). Similarly, infecting tomatillo (*Physalis philadelphica*) plants with recombinant TMV-expressing RNAi effectors also resulted in a decrease in *Bactericera cockerelli* progeny production after feeding (Wuriyanghan and Falk, 2013). In another study, researchers engineered *Citrus tristeza virus* (CTV), a common virus of citrus, with *D. citri* truncated *abnormal wing disc* (*awd*) RNA sequence triggering *awd* gene silencing after *D. citri* nymphs fed on infected plants, causing wing malformation and mortality in adult insects (Hajeri et al., 2014). The *Potato virus X* (PVX) engineered with *Bursicon* and *V-ATPase* gene sequences significantly reduced the population of the cotton mealybug (*Phenacoccus solenopsis*) after insects fed on *Nicotiana tabacum* plants inoculated with the recombinant PVX (Khan et al., 2018). Furthermore, insect-specific viruses can be exploited as VIGS vectors to control insect pests (Kolliopoulou et al., 2017; Nouri et al., 2018). For instance, researchers investigated the ability of engineered *Flock house virus* (FHV) to induce gene suppression through RNAi in S2 cells derived from *D. melanogaster* embryos and insects at the adult stage. The recombinant FHV carrying the target gene sequences caused significantly higher mortality (60–73% and 100%) than the wild type virus (24 and 71%) in both S2 cells and adult insects, respectively (Taning et al., 2018).

To date, the sources of RNA-based molecules (dsRNA or siRNA) commonly utilized in insect and disease management studies are costly synthetic molecules or are produced through time-consuming, laborious procedures. To overcome the shortages of these methods, the potential of delivering dsRNA expressed in bacteria has been investigated, providing an alternative method for large-scale target gene screening (de Andrade and Hunter, 2016; Zotti et al., 2017). In Lepidoptera, the cotton bollworm (*Helicoverpa armigera*) larvae exposed to an artificial diet coated with engineered bacteria for five days showed high mortality and inhibition in the expression levels of target genes, causing drastic reductions in body weight, body length, and pupation rate (Ai et al., 2018). Oral toxicity of *Escherichia coli* expressing dsRNA targeting the *integrin β1 subunit* was observed in *Spodoptera exigua* larvae; this resulted in insect mortality, damage to the midgut epithelium tissue, exhibition of a marked loss of cell-cell contact, and remarkable cell death, which further resulted in increased susceptibility to a Cry insecticidal protein from *B. thuringiensis* (Kim et al., 2015). Also, the growth and development of *S. exigua* larvae fed with *E. coli* expressing dsRNA targeting *chitin synthase A* was disturbed, resulting in mortality (Tian et al., 2009). Moreover, in the gypsy moth (*Lymantria dispar*), a serious insect pest of the North American forests, bacterial expression of dsRNA resulted in target-gene knockdown and a subsequent reduction in body mass and egg masses (Ghosh and Gundersen-Rindal, 2017). In the oriental armyworm (*Mythimna separate*), a study showed that oral delivery of bacterially expressed dsRNA led to RNAi effects, with knockdown of target genes, reduction of body weight, and increased mortality (Ganbaatar et al., 2017). In Diptera, *Bactrocera dorsalis* adults fed on an artificial diet coated with *E. coli* expressing dsRNA exhibited a reduction in target gene mRNA levels and a reduction in egg-laying (Li et al., 2011). In Coleoptera, the potential of feeding dsRNA expressed in bacteria to manage populations of Colorado potato beetle (*L. decemlineata*) was observed due to the resulting knockdown of five target genes tested, which caused significant mortality and reduced body weight gain in treated beetles (Zhu et al., 2011).

Besides the use of bacteria as a dsRNA delivery method to pests, these microorganisms have been used to produce large amounts of dsRNAs, which can be sprayed on crops at any time with lower costs (Joga et al., 2016). For example, the *E. coli* HT115 (DE3) strain has been used to produce large amounts of dsRNA since it lacks the enzyme that degrades dsRNAs (Papic et al., 2018; Ahn et al., 2019). Also, studies have shown the efficiency of dsRNA

produced in bacteria to control plant viruses (Robinson et al., 2014; Mitter et al., 2017b). Crude extracts of *E. coli* HT115 containing dsRNA targeting the *Sugarcane mosaic virus* (SCMV) *coat protein* gene were used in maize plants as a preventive spray and they inhibited the SCMV infection (Gan et al., 2010). Other works reported the use of bacteria to produce dsRNAs from *Pepper mild mottle virus* (PMMoV), PPV, and TMV to protect plants against these pathogens. The application of crude bacterial preparation via spray onto tobacco plant surfaces provided protection against infection from these viruses (Tenllado et al., 2003; Yin et al., 2009). Moreover, this system of dsRNA production in bacteria can deliver multiple virus dsRNAs to disrupt several virus species at once and may achieve multiple virus resistances at one time (Tenllado and Díaz-Ruíz, 2001; Yin et al., 2009).

Recently, advances in sequencing technology and the characterization of insect gut microbiota are leading to the identification of novel symbiotic microorganisms suitable to be genetically modified and used as dsRNA delivery vectors to control insects (Krishnan et al., 2014). Using symbiont-mediated RNAi is an intriguing strategy in which the relationship between culturable symbiotic gut bacteria, or yeast, and the host can be exploited in order to constitutively produce dsRNA to induce RNAi in the host, and the use of symbiotic bacteria has been shown to be a promising delivery strategy to control insects (Abrieux and Chiu, 2016; Joga et al., 2016; Whitten and Dyson, 2017). Also, dsRNA can be delivered into target pests through the infection of entomopathogenic fungus and may result in the development of a new RNAi methodology for pest control. For instance, the application of *Isaria fumosorosea*, a common fungal pathogen of the B-biotype *Bemisia tabaci*, expressing dsRNA of whitefly immunity-related genes, resulted in knockdown of the target gene and increased whitefly mortality (Chen et al., 2015).

Although viruses and bacteria, following genetic modification to express dsRNA and induce gene silencing, are promising strategies to deliver dsRNA in the field, they will be considered as GM products and will suffer the same regulatory and public acceptance obstacles as GM crops.

Other Applications

In relation to the natural role of RNAi to protect cells from virus infections, this technology could be used to protect beneficial insects, such as bees, from viral diseases. In 2010, large-scale field trials tested the efficiency of Rembee™ (Beeologics, LLC, Miami, FL, USA), a dsRNA product designed to protect honeybees (*Apis mellifera*) from *Israeli acute paralysis virus* (IAPV) infection (Hunter et al., 2010). The product successfully protected the hives from the virus infection, resulting in several bees that were twice as large in the dsRNA-treated hives compared to untreated. As a result, dsRNA-treated hives produced three times as much honey compared to untreated ones. In another study, a similar result was observed in bumblebees (*Bombus terrestris*), which upon being fed on IAPV virus-specific dsRNAs, showed decreased mortality (Piot et al., 2015). In other studies carried out on *A. mellifera*, RNAi was also efficient against the internal microsporidian parasite *Nosema* (Paldi et al., 2010; Rodríguez-García et al., 2018) and the obligatory ectoparasite *Varroa destructor* (Garbian et al., 2012). The control of these organisms, which are associated with colony decline, improved the health of hives and shines a light on the development of effective treatment alternatives for diseases in bees and other beneficial insects in the future.

ISSUES INVOLVING NON-TRANSFORMATIVE DELIVERY APPROACHES

In the near future, the exogenous application of RNA molecules to induce RNAi-mediated gene silencing will influence the traditional way we protect crops from insects and pathogens. Due to uptake restrictions, it is believed that the development of RNA-based products will focus on the use of dsRNA as the molecule to induce gene silencing (Sammons et al., 2011). The minimum required length of a dsRNA to achieve an RNAi effect will vary depending on target genes and species (Bolognesi et al., 2012). Consequently, the formulations can contain only one dsRNA molecule, be a combination of short and long dsRNAs targeting one or more genes, or otherwise be a combination of dsRNA and insecticide or fungicide, managing a resistant population and reaching better results.

Under field conditions, RNA-based biopesticides would need periodical applications following plant growth to ensure plant protection. Also, while the RNA-based products are a new and highly specific mode of action, the timing issues of "when should I spray?", a dilemma that growers already have with current chemical control approaches, is also something that needs to be studied and understood. Although the vascular system of plants translocate RNAs (Melnyk et al., 2011), allowing RNA molecules to travel across long distances inside the plant and protecting untreated areas, the necessity of reapplication implies an increase in cost. Thus, it is expected that, with the use of non-transformative strategies to control insects and pathogens, the dsRNA molecule will remain active long enough to effectively control the target pest. Moreover, although selection of the most effective target gene is desirable, even partial suppression can cause severe damage and irreversible lethal effects (Huvenne and Smagghe, 2010). Transient effects of this technique should not be an overwhelming drawback to the use of non-transformative approaches. In addition to this, the development of more efficient dsRNA mass production systems will reduce costs and, together with the release of new formulation strategies, will allow foliar spray, trunk injection, and irrigation, among other approaches, to be exploited as potential control strategies (Hunter et al., 2012; de Andrade and Hunter, 2016).

DsRNA production costs have been dropping significantly over the last years, from ~ $12,500 USD per gram in 2008 to less than $60 USD per gram in 2018 (Cagliari et al., 2018), with an expectation of further significant reduction in prices in the next years. Mass dsRNA production systems, such as *in vitro* or *in vivo* production systems, allow high dsRNA production with the reduction in costs. These are strategies based on the hybridization of two single-stranded RNAs (ssRNAs), enzymatically synthesized, which can be performed *in vitro* (Tenllado and Díaz-Ruíz, 2001; Koch et al., 2016; Konakalla et al., 2016; Wang et al., 2016b) or *in vivo* (using bacterial cells

deficient of enzyme RNase III that degrades dsRNAs) (Tenllado et al., 2003; Gan et al., 2010). Although an *in vivo* system allows for the production of bulk amounts of dsRNA compared to *in vitro* synthesis, it still results in high cost, hard purification, and high labor demand (AgroRNA, http://www.agrorna.com/sub_02.html), and, after all, is still naked dsRNA that under field conditions presents a shorter half-life. Thus, dsRNA formulation is a promising alternative to increase stability and boost the efficiency of gene silencing in recalcitrant species in Lepidoptera and Hemiptera, allowing plants to be protected for longer.

The technology "BioClay," a layered double hydroxide (LDH) clay nanosheet, provided high dsRNA stability under field conditions, increasing the residual period of dsRNA on plants and protecting them from virus infection for up to 30 days compared to naked dsRNA (Mitter et al., 2017a). Guanylate Polymers increased RNAi efficiency in *S. exigua* (Christiaens et al., 2018b) and *Spodoptera frugiperda* (Parsons et al., 2018), and they pave the way for future applications of RNA-based pest control strategies in lepidopteran insects. This technology is based on the use of formulations to enhance stability of dsRNA in insects. Encapsulation of dsRNA molecules in liposome complexes also increased dsRNA stability and enhanced cellular uptake in Dipteran insects (Whyard et al., 2009; Taning et al., 2016) and Blattodea (Lin et al., 2017). In *Euchistus heros*, liposome complexes increased nymph mortality compared to naked dsRNA (Castellanos et al., 2018). However, in some cases, even with the use of formulation the dsRNA molecules were unable to initiate the RNAi process. This was the case in the migratory locust (*Locusta migratoria*), where liposome encapsulation was not efficient to protect the dsRNA, leading to inefficient RNAi in this species (Luo et al., 2013).

Considering the hostile environmental conditions to which dsRNA molecules are exposed in the field, a biotechnology company called RNAagri (former APSE) developed a system where APSE RNA Containers (ARCs) are produced by *E. coli* bacteria, allowing for the mass production of encapsulated ready-to-spray dsRNA (APSE technology; www.apsellc.com). This technology is based on bacteria engineered with a plasmid to produce naturally occurring proteins such as capsids, which are then co-transformed with another plasmid coding for the target dsRNA or siRNA together with a sequence called the "packing site". The double-transformed *E. coli* are then purified, resulting in self-assemble particles that have encapsulated the desired RNAs. These particles protect the RNAs and enhance resistance to adverse environmental conditions, and, once sprayed, they are expected to be taken up by the insect rapidly (Kolliopoulou et al., 2017). The development of formulations to carry dsRNA efficiently up to the target organism is of paramount importance to the success of developing non-transformative strategies for pest control, and advances in this area in the future will boost the use of these strategies.

Successful cases using foliar spray, irrigation, and trunk injection have already been reported (**Table 3**), but the application range may be much broader. The choice of the dsRNA delivery strategy is of great importance in the development of non-transformative delivery methods, and it will vary according to the target pest and crop. RNAi efficiency naturally varies among the target species, life stage, and delivery strategy, and the choice of a correct combination of these factors will save years of research and resources. Regardless of the delivery strategy or target species, for a successful non-transformative RNAi strategy it is also of paramount importance to identify unique regions in essential target genes so that little changes in expression level will provoke severe consequences. For example, foliar application of dsRNA was unable to induce the RNAi machinery in *T. vaporariorum* due to the low dsRNA uptake by the insects (Gogoi et al., 2017). In order to achieve success using RNAi-based gene silencing as a control strategy, low amounts of RNA molecules need to be enough to trigger the machinery and lead to insect or pathogen mortality. In insects, screening for target genes through artificial diet containing dsRNA is an easy procedure to screen large numbers of dsRNA molecules, resemble field conditions (Araujo et al., 2007; Whyard et al., 2009; Aronstein et al., 2011), and address important issues such as better target genes, effective dsRNA, and effective lethal concentration (LC50) (Araujo et al., 2007; Baum et al., 2007; Bachman et al., 2013). However, under field conditions it is difficult to establish the amount of dsRNA taken up by the target pest, which hinder determination of the LC50.

Coleopteran insects are considered very susceptible to RNAi (Baum et al., 2007; Baum and Roberts, 2014), while insects in the order Lepidoptera are considered recalcitrant and high dsRNA concentrations are required to achieve successful gene silencing results (Terenius et al., 2011). Limiting factors, such as dsRNA degradation (Wang et al., 2016a; Guan et al., 2018) and the entrapment of internalized dsRNA in endosomes (Yoon et al., 2017), have recently been associated with unsuccessful RNAi (Niu et al., 2018). In some hemipteran insects, such as *Acyrthosiphon pisum*, the lack of response under dsRNA supply is also associated with high nuclease activity (Christiaens et al., 2014). Thus, we believe significant advances in dsRNA formulation will occur in the next years, and so the development of RNA-based non-transformative products will be focused on non-recalcitrant groups.

Another important point in the use of non-transformative strategies for RNA delivery, mainly *via* foliar application, is that, during the application, not only the target pest will receive the RNA molecules, but also non-target insects. In GM plants, researchers have shown that expressed dsRNA has a high degree of specificity towards control insects (Dillin, 2003; Whyard et al., 2009; Petrick et al., 2013) or pathogens (Koch et al., 2013). However, other studies have shown that siRNAs can knockdown non-target genes (Birmingham et al., 2006). In mammals, studies have shown that even with differences between the nucleotide sequences from siRNA and the target mRNA gene silencing still occurs (Jackson et al., 2003; Schwarz et al., 2006; Huang et al., 2009). However, there is no consensus among scientists on the number of nucleotides from the siRNA that must match the target sequence identically, and more research is needed to determine if the same issues found in mammalian cells apply to other organisms such as insects or pathogens (Christiaens et al., 2018a). Therefore, target regions and dsRNA molecule design is very important. Baum et al. (2007) tested the specificity of dsRNA molecules based on the identity of the nucleotide sequence of the *V-ATPase* gene *subunits A* and *E*

between *D. v. virgifera* and *L. decemlineata*. The target sequences of the *V-ATPase subunit A* shared 83% identity, while the target sequences of the *V-ATPase E* subunit of these insects shared 79% identity. Feeding both *D. v. virgifera* and *L. decemlineata* with the non-specific dsRNAs caused mortality in both species (Baum et al., 2007). However, researchers already expected this response as most of the ~21 nt siRNAs obtained would have a similarity to both species, causing non-specific silencing. GM tobacco plants expressing a dsRNA targeting the *EcR* gene in *H. armigera* were also effective against another lepidopteran pest, *S. exigua* (Zhu et al., 2012). The target sequence of both species had a high similarity in the nucleotides sequences (89%), and, when both species fed on the GM tobacco plants, this resulted in mortality levels between 40–50%. However, when the necessary care at the time of dsRNA design is taken, it is possible to obtain extremely specific or broad-range molecules. To show the specificity of dsRNA-based gene silencing, the molecules were designed to target the *V-ATPase* gene in four different species, *D. melanogaster* (Diptera), *Tribolium castaneum* (Coleoptera), *A. pisum* (Hemiptera), and *M. sexta* (Lepidoptera), resulting in target gene silencing with no effects over non-target species (Whyard et al., 2009). They also demonstrated the feasibility of designing specific dsRNA molecules even within species of the same genus. Hence, the design of the dsRNA will determine the action spectrum of the molecules; molecules with a larger action spectrum are not necessarily harmful. If carefully designed, broad-spectrum RNA-based molecules can be used to protect plants against diverse insects and pathogens.

PERSPECTIVES IN A GLOBAL VIEW

During the last decade, significant advances have been made to find better ways to control insects and pathogens in crops, reduce environmental impacts, and improve profits. Scientists have harnessed technologies such as RNAi-based gene silencing to turn off essential genes in target organisms, leading to mortality. Studies using foliar applications, trunk injection, and irrigation have demonstrated the feasibility and efficacy of RNAi-based gene silencing through non-transformative delivery strategies (**Table 3**). Other delivery methods still need to be investigated, such as seed coats or baits. To our knowledge, no studies for the development of RNA-based products as seed coat or powder/granules formulations are available. While the main objective of the seed coat is to protect plants from the attack of insects and pathogens during the initial growth phase, powder/granules formulations could be applied on the soil or substrate surface. Similarly, the use of baits (spray or station) containing RNA is a promising non-transformative delivery strategy that could be developed for pest control, especially in orchards. The bait spray can consist of an attractant mixed with a specific RNA, while bait stations can be containers with sRNA molecules and attractants, which will attract the pest to the bait. These are techniques that can be explored further in the use of RNAi in crop protection.

RNA biopesticides are compounds occurring naturally in the environment and inside organisms and are thus potentially less harmful than synthetic pesticides. These molecules are naturally internalized by eukaryotic organisms, subject to RNAi pathways, and degraded by natural cellular processes. Also, dsRNAs are rapidly degraded when present in water or soil (Dubelman et al., 2014; Albright III et al., 2017; Fischer et al., 2017; Parker et al., 2019), reducing the chances to leave residues in the environment or food products. As with any control method, targeted insects, pathogens, and viruses can develop resistance.

The use of genomic tools will allow the development of technologies such as RNA-based products to increase crop resistance against insects, pathogens, and viruses. Also, the development of RNA formulations will improve RNAi efficiency and field stability. So, these could even replace chemical pesticides in some applications or, when in combination, reduce the use of chemical pesticides at least.

AUTHOR CONTRIBUTIONS

DC, ND, GS, and MZ contributed to the conception of the manuscript. DC and ND wrote the first draft. DC, ND, DG, ES, GS, and MZ wrote sections of the manuscript. GS and MZ revised and edited the manuscript. All authors read, contributed critically to the drafts, and approved the final version.

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RNAiSeq: How to See the Big Picture

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Targeting genes *via* RNA interference (RNAi) has become a successful method to reduce pest populations. Ideally, the expression of a gene critical for a life function in the insect is targeted by specific dsRNA, *via* spray or oral delivery. Experts have developed working guidelines in the development and regulation of RNAi as a pesticide. We argue that an important tool in the validation of RNAi is genome-wide expression analysis in the targeted pest, and we name this approach RNAiSeq. We have used RNAiSeq in the coleopteran model *Tribolium castaneum* to validate knockdown of target genes, and to examine the effect of knockdown on other genes. With RNAiSeq, we identified compensation responses to the knockdown of a gene encoding a major digestive enzyme in larvae that correlated to the responses we have observed with ingested protease inhibitors. Compensation can mask RNAi phenotypic responses and is important to understand in the context of efficacy. RNAiSeq also has identified new gene interactions that were previously unassociated with the target gene, important in the context of the large number of genes without associated functions in insects and other organisms. We discuss other research where RNAiSeq has led to important findings. These data not only provide validation of target knockdown, but also further identify changes in the expression of other genes impacted by the knockdown. From the context of pest control, this information can be used to predict genetic changes that will impact the efficacy of RNAi products in target pests.

Keywords: gene expression, RNASeq, stored product insect, topical RNAi, *Tribolium castaneum*, pest control product

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INTRODUCTION

RNA interference (RNAi) is one of the mostly widely used tools to study gene function in insects. RNAi is also a potential control product being developed to combat problem pests *via* either oral or topical application. We and others have discussed the benefits and problems associated with RNAi (Baum et al., 2007; Noh et al., 2015; Joga et al., 2016; Perkin et al., 2016b). Our research has focused on developing new insect control products, including RNAi, for stored product beetles.

Our model for the development of RNAi for stored product pest control is the red flour beetle, *Tribolium castaneum* (**Figure 1**). This insect feeds on grains and stored products and inflicts major economic damage worldwide (Pimentel, 1991). Flour beetles are responsible for losses in stored grains, warehouses, and flour mills, among others. Fumigants such as phosphine are the most common control product, but many

FIGURE 1 | *Tribolium castaneum* adult feeding on grain (photo courtesy USDA ARS, Peggy Greb).

populations of phosphine-resistant beetles and other stored product insects have been identified (Pimentel et al., 2010; Opit et al., 2012).

T. castaneum has served as a genetic model for coleopteran research and was the first agriculturally important insect to have a sequenced genome (Tribolium Genome Sequencing Consortium, 2008). *T. castaneum* has a robust response to injected RNAi (Brown et al., 1999; Tomoyasu and Denell, 2004; Aronstein et al., 2011; Miller et al., 2012), but success with oral RNAi has been documented in only a few studies (Whyard et al., 2009; Cao et al., 2018). We and others have not observed a phenotype or mortality response to oral RNAi and have postulated that the problem is either nucleases in the alimentary canal, or lack of transport in the gut (Palli, 2014, unpublished data).

Here we discuss the value of using RNA-Seq as a validation tool for RNAi, which we refer to as RNAiSeq, and we demonstrate the value of RNAiSeq in case studies from our research. We also demonstrate how RNAiSeq has been used in other organisms to make important discoveries.

CASE STUDIES

Our Case Studies

In our first case study (**Figure 2**), we sought to understand the effect of knockdown of a major digestive enzyme. Cysteine peptidases are major digestive enzymes in the anterior midgut of *T. castaneum*, but they also have other physiological roles in the insect (Vinokurov et al., 2009; Perkin et al., 2016a). In the functional characterization of cysteine peptidases in *T. castaneum*, we found that one gene, *TC01101*, encoded the primary cysteine peptidase and was the mostly highly expressed gene in the larval gut (Morris et al., 2009; Perkin et al., 2016a). We used RNAiSeq to investigate the effect of knockdown of *TC01101* using dsRNA designed from the 3' end, 5' end, middle, and entire sequence (Perkin et al., 2017a). All constructs resulted in significant reduction in *TC01101* gene expression. However, other cysteine peptidase genes were increased in expression, effectively compensating for the loss of *TC01101* and masking any loss-of-function effects of the target gene. This compensation response also was accompanied by increased expression of serine peptidase genes. Importantly, these responses paralleled the compensation we had observed at the protein level in insects fed protease inhibitors (Oppert et al., 1993, 2005, 2010). The data provided crucial molecular evidence to explain how insects adapt to and survive on diets containing protease inhibitors through the regulation of an intricate network of duplicated genes. The remaining piece of the puzzle is to identify the regulatory elements responsible for the adjustments in gene expression to compensate for inhibitors or other disruptive dietary compounds (such as dsRNA), research in progress. However, the data demonstrate an evolved and elegant feedback mechanism to conserve digestive efficiency in this stored product beetle.

In the second case study (**Figure 3**), we evaluated the effect of knockdown of a gene used as a positive phenotypic control. In our early experiments with *T. castaneum*, we commonly used a positive injection control, *aspartate 1-decarboxylase* (*ADC*), because it provided a visual confirmation of knockdown. *ADC* is involved in the cuticle tanning pathway that produces a red phenotype (Arakane et al., 2009), and reduced expression of *ADC* results in a black beetle. RNAiSeq validated the significant knockdown of *ADC*, but it also uncovered a change in the expression of other metabolic genes (Perkin et al., 2017b). These changes included decreased expression of odorant receptors and allatotropin genes, but highly increased expression of *dopamine receptor 2*. In *Drosophila melanogaster*, the increased expression of a dopamine receptor was linked to reduced mobility (Phillips et al., 2005). Therefore, we hypothesized that beetles injected with *ADC*-dsRNA would have slower movement due to increased expression of *dopamine receptor 2*. In fact, dsRNA-injected beetles moved slower and over shorter distances than noninjected control beetles. Therefore, RNAiSeq provided insight into a previously unknown interconnected pathway between *ADC* and *dopamine receptor 2*.

In our last case study (**Figure 4**), we evaluated the effects of the knockdown of a unique cuticle protein gene (*CPG*)

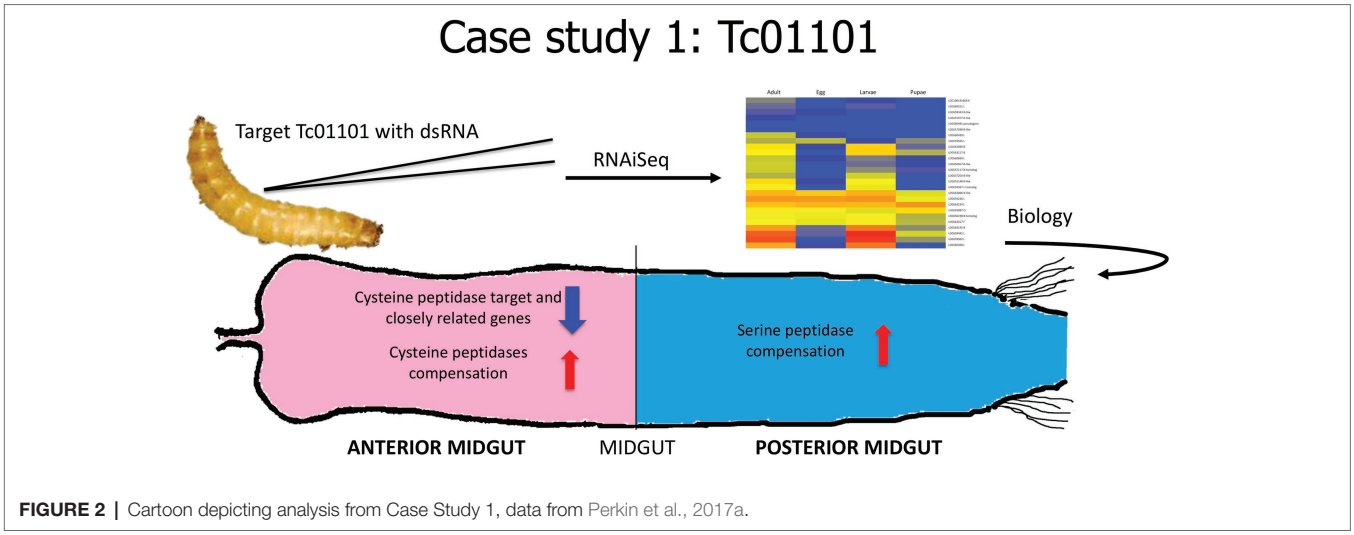

FIGURE 2 | Cartoon depicting analysis from Case Study 1, data from Perkin et al., 2017a.

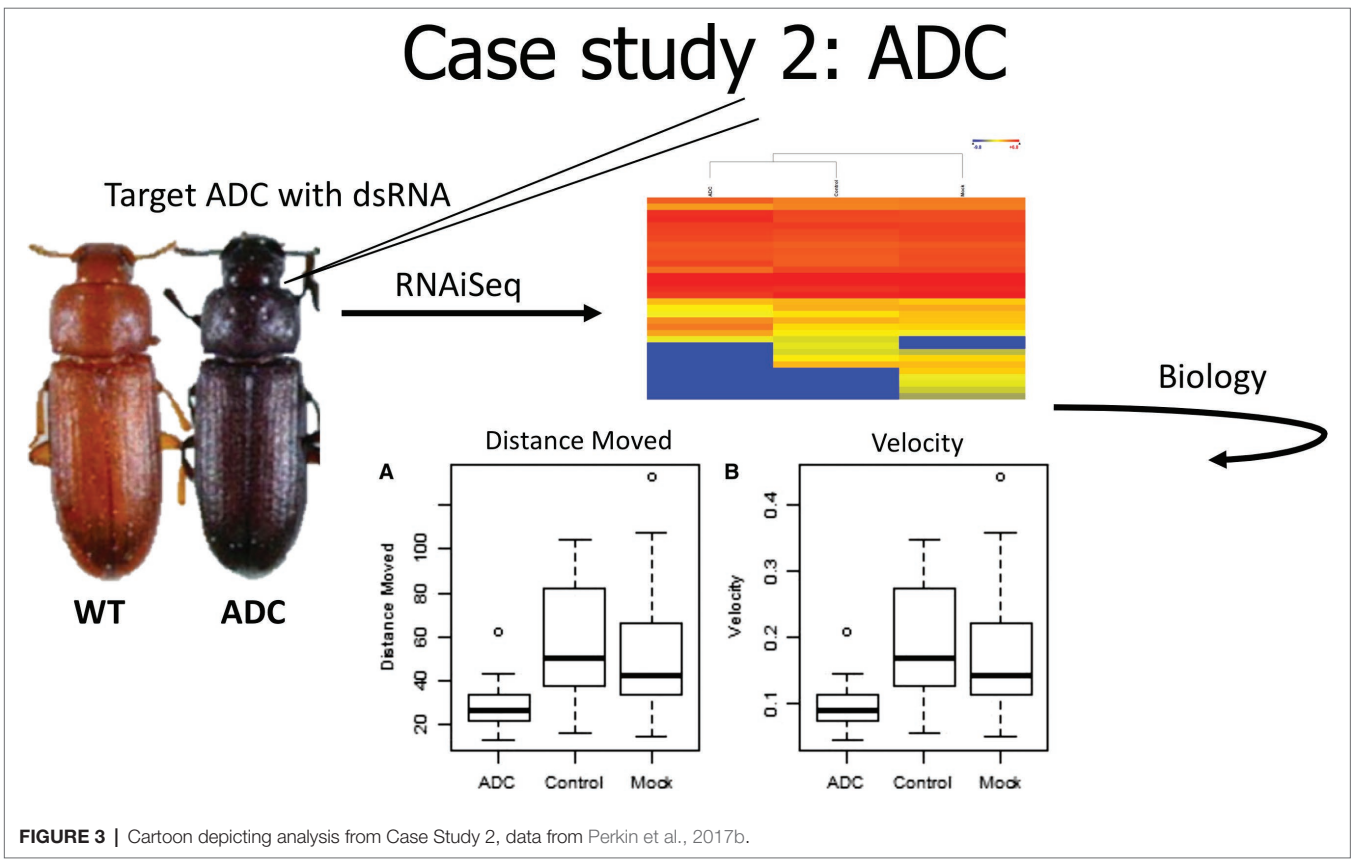

FIGURE 3 | Cartoon depicting analysis from Case Study 2, data from Perkin et al., 2017b.

found in larvae. Knockdown of *CPG* in *T. castaneum* larvae resulted in significant mortality, and RNAiSeq validated target knockdown as well as the discovery of compensation responses of other cuticle protein genes (Perkin and Oppert, 2019). Interestingly, *CPG* knockdown in *T. castaneum* also induced significant ($p < 0.01$) differential expression of 52 long noncoding RNAs (lncRNAs). Because lncRNA can induce epigenetic changes that alter gene transcription, including silencing (Tufarelli et al., 2003), we propose that these lncRNAs may be involved in the altered expression of *CPG* and related genes. The role of lncRNA in gene silencing mechanisms warrants further research.

We compared the expression of genes that are typically associated with RNAi in other insects from these three studies

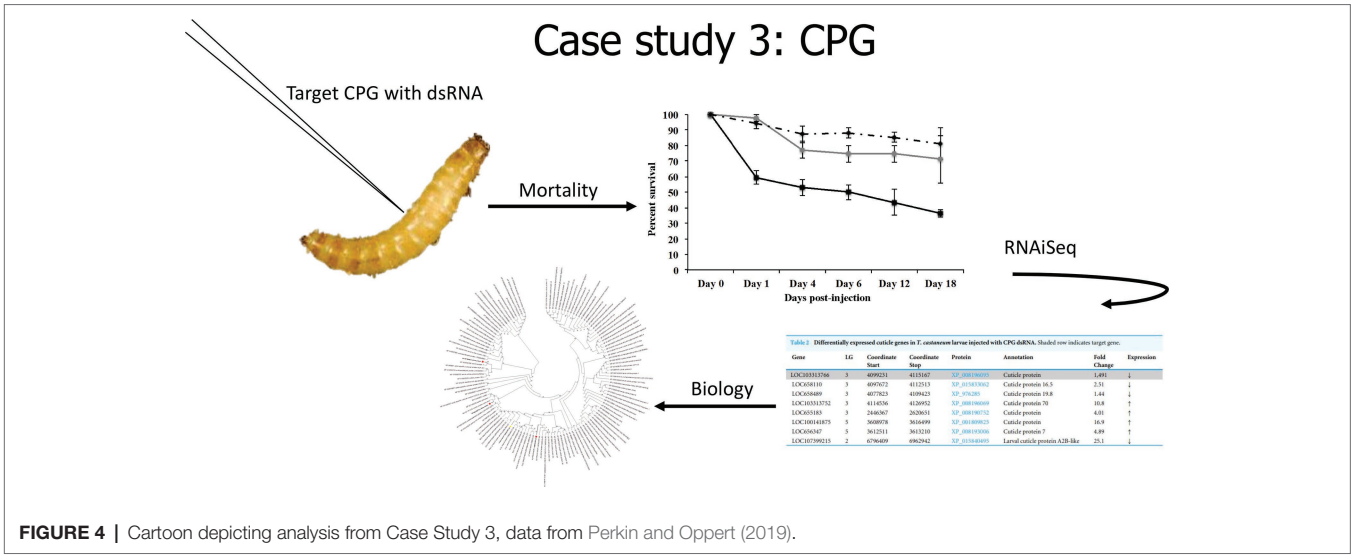

FIGURE 4 | Cartoon depicting analysis from Case Study 3, data from Perkin and Oppert (2019).

to determine if patterns could be observed in response to injected dsRNA in *T. castaneum* (**Figure 5**). While only some of the comparisons were statistically significant, the overall trend was that most of the RNAi genes were increased in expression in larvae that were injected with dsRNA. The greatest increase in expression was observed with Ago-1 and Ago-2a (up to 5-fold increase) in the CPG study. The data may reflect the upregulation of RNAi systems in the cell, but more research is needed to determine the mechanisms of increased expression and implications for gene silencing.

Other Case Studies

An important discovery in understanding the conservation of DNA methylation in eukaryotes was made by RNAiSeq in honeybees. Previously, it was demonstrated that RNAi silencing of *DNA methyltransferase 3* (*dnmt3*) increased the number of worker larvae developing into queens (Kucharski et al., 2008). By combining a unique delivery method for dsRNA (aerosol application through the trachea) and RNASeq and software to identify alternative splicing (Li et al., 2013), four different types of splicing events were detected in response to *dnmt3* gene knockdown (Li-Byarlay et al., 2013). Both exon skipping and intron retention were correlated to decreased methylation from a loss of *dnmt3* function. The data convincingly supported the hypothesis that DNA methylation of genes can regulate alternative splicing, an important finding on how environmental cues can affect gene expression.

In another study, loss of function in cultured cells by either RNAi, antisense oligonucleotides, or CRISPR genome editing of lncRNA or protein coding genes was compared *via* RNAiSeq (Stojic et al., 2018). All methods effectively reduced transcription of the target, but they also induced off target effects. Notably, all methods resulted in differences in both molecular and cellular phenotypes. It was recommended that multiple targeting sequences be evaluated by RNASeq with proper controls that are also compared to untreated samples. Recommendations also included titrating the amount of product (i.e., dsRNA) to use the minimal dose required for a response to reduce off target effects, but this has not been evaluated empirically and may not be practical for the development of products for insect control.

DISCUSSION

Our experience with combining RNAi and RNASeq, which we now refer to as RNAiSeq, accomplishes the primary task of verification of knockdown of the target gene, but also has yielded unexpected discoveries of gene function and metabolic interactions. We propose that incorporating RNAiSeq into the development of new insect control products, including topical RNAi, provides valuable insights into the response of the transcriptome due to the loss of function from the target gene. This information can be used to increase the potency of the dsRNA by adjusting the dose, choosing a different region of the target DNA, or adjusting the experimental design to avoid reduced product efficacy through compensation responses.

The differential expression of genes other than the target genes is likely not due to direct degradation of transcripts. In most cases, we suspect that the loss of the gene product provides a regulatory response that either decreases or increases the expression of other genes functioning in a network. Exceptions in our studies were likely found in some of the compensation responses of genes that were highly homologous through gene duplication, including genes encoding cysteine proteases or cuticle proteins. Although we sought regions that were unique in the primer design for our dsRNA constructs, it is possible that smaller siRNAs from DICER may have directly interacted with nontarget genes. Bioinformatics is also highly dependent on the accuracy of predicted gene sequences.

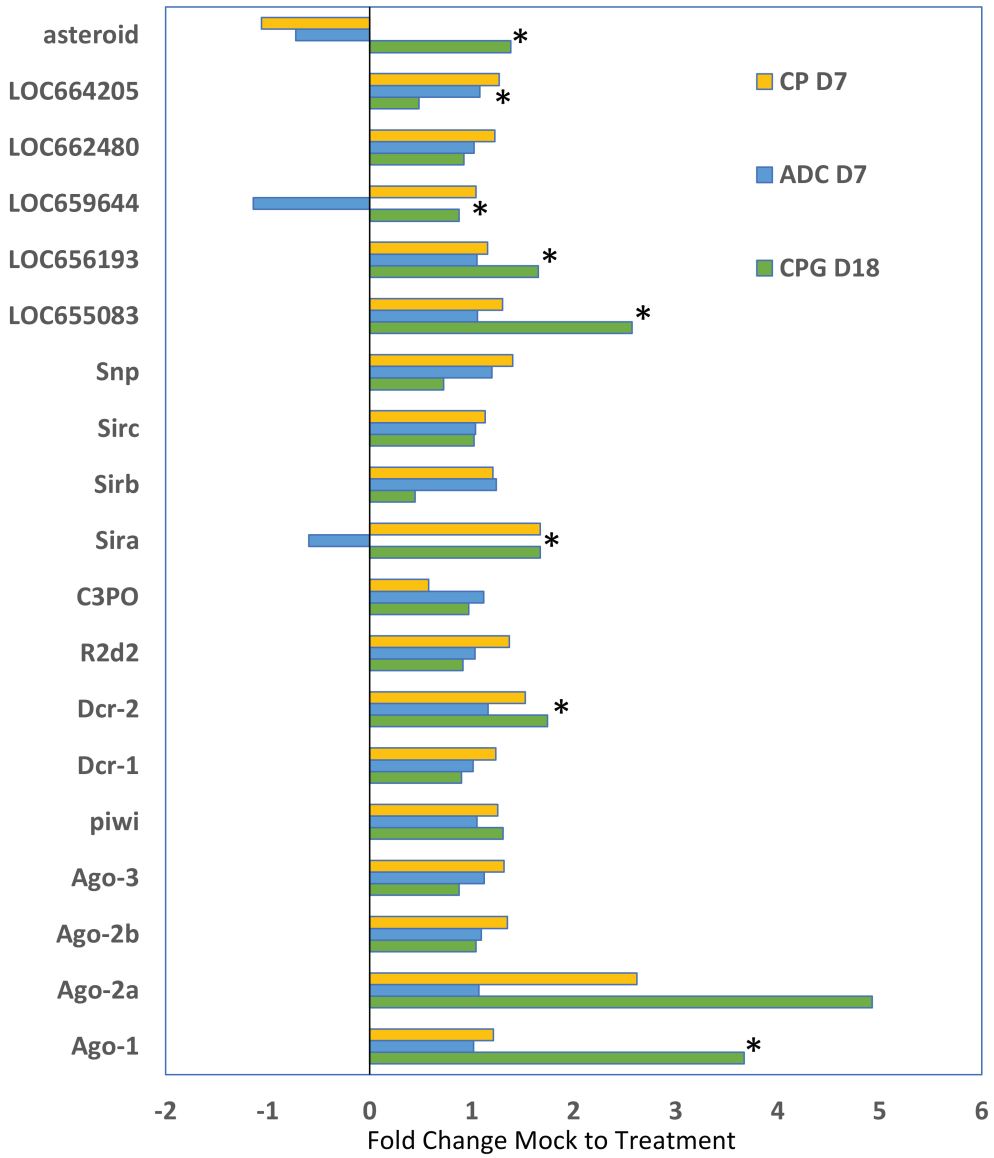

FIGURE 5 | Differential expression of genes typically involved in RNAi (identified on the y axis) as determined by the fold change difference of treatment expression versus that of the control mock injected. CP D7, data from the cysteine peptidase study, analyzed at day 7 post injection (Perkin et al., 2017a); ADC D7 data were from Perkin et al. (2017b) analyzed at day 7 post injection; CPG D18 data were from Perkin and Oppert (2019) analyzed at day 18 post injection. Data that were significantly different ($p < 0.05$) indicated by asterisk.

Selection of the timepoint(s) for RNAiSeq may need to be verified experimentally. We routinely use day 7 post injection for extraction of RNA, but we extended the timepoint to day 18 in the CPG study (Perkin and Oppert, 2019) due to delayed effects on mortality. Additionally, we used whole larvae in our experiments to observe global transcriptome responses in the whole animal, but RNAiSeq could be used for selected tissues or even single-cell transcriptomics, provided the amount of material is sufficient for libraries. Of course, other techniques, such as proteomics, will provide even more supplemental information on the effects of gene knockdown.

We anticipate that the routine use of RNAiSeq will yield additional important benefits. Wood et al. (2019) posed the exciting paradox that while most of the functional discoveries in yeast proteins were made during the 1990's, we still have little knowledge of the function of 20% of protein coding genes. Orthologs with unknown function are maintained in the genomes of other organisms, including insects, but what are these highly conserved mystery gene products doing? Domains of unknown function (DUFs) are increasingly implicated in niche roles, such as the discovery of DUF1220 copy number in the severity of autism (Davis et al., 2019). Discovery of protein functions unique to insects also are

important in understanding the biology of the organism and crucial to pest control. Alternatively, these unique proteins are finding novel applications, such as the incorporation of spider silk proteins into industrial products (Römer and Scheibel, 2008).

Our studies demonstrate that even with genes that have well-defined functions, additional functions can be identified through observation of transcriptome responses to target gene loss of function or reduced expression. Many genes function in intricate networks, often multiple networks, and defining interconnections in networks can lead to more accurate predictions in disrupting gene function. Ultimately, accurate mapping of these networks will lead to a better understanding of the biology of the organism and provide better tools to combat pests and disease.

AUTHOR CONTRIBUTIONS

BO and LP were involved in all aspects of the case studies and also contributed to the presentation and this manuscript.

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Environmental Fate and Dissipation of Applied dsRNA in Soil, Aquatic Systems, and Plants

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Two primary use patterns exist for dsRNA-based products for crop protection: *in planta* produced dsRNA such as in a genetically engineered (GE) crop; and topically applied dsRNA such as a spray application. To enable effective environmental risk assessments for these products, dsRNA must be successfully measured in relevant environmental compartments (soil, sediment, surface water) to provide information on potential exposure. This perspective reviews results from numerous environmental fate and degradation studies with topically applied unformulated dsRNAs to demonstrate the high lability of these molecules and low potential for persistence in the environment. Additionally, we report on results of a pilot study of topically applied dsRNA on soybean plants demonstrating similar rapid degradation under field conditions. Microbial degradation of nucleic acids in environmental compartments has been shown to be a key driver for this lack of persistence. In fact, the instability of dsRNA in the environment has posed a challenge for the development of commercial topically-applied products. Formulations or other approaches that mitigate environmental degradation may lead to development of commercially successful products but may change the known degradation kinetics of dsRNAs. The formulation of these products and the resultant impacts on the stability of the dsRNA in environmental compartments will need to be addressed using problem formulation and product formulation testing may be required on a case by case basis to ensure an effective risk assessment.

Keywords: RNAi, dsRNA, environmental risk assessment, environmental fate, dissipation

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INTRODUCTION

To conduct an effective environmental risk assessment (ERA) for a dsRNA-based, pesticidal agricultural product, it is necessary to determine the routes of exposure for non-target organisms (NTOs) and reliably quantify the concentration and persistence of the dsRNA in relevant environmental compartments such as plant tissues, soil, and surface waters/sediment. Two primary use patterns exist for dsRNA-based products in crop improvement: *in planta* produced dsRNA such as in a genetically engineered (GE) crop; and topically applied dsRNA such as a spray application.

As discussed in Romeis and Widmer (2019), problem formulation is a core component of the ERA framework offering a logical approach and roadmap to characterize risk. Key to this approach is defining assessment endpoints, developing a conceptual model of predicted environmental relationships, and drafting an analysis plan to collect relevant data in regard to exposure and effects to perform a risk characterization (Nickson, 2008).

This perspective summarizes the current research on the environmental fate and degradation of dsRNA, with a focus on topically applied dsRNA, including exposure scenarios and quantification approaches, as well as identifying gaps in knowledge and key questions to be addressed in ERAs for dsRNA crop protection products.

EXPOSURE SCENARIOS

For *in planta* expressed dsRNA the concentration of dsRNA across tissues and growth stages can be used to estimate the maximum exposure levels to terrestrial and aquatic NTOs. Typically, samples are collected from multiple tissues (e.g. pollen, leaf, root) and analyzed across life stages of the plant to provide a thorough characterization of the expression of the dsRNA as NTOs may feed on or be exposed to specific plant tissues at specific life stages of the plant. The primary receiving compartment for *in planta* produced dsRNAs is the soil due to the incorporation of plant biomass post-harvest. Based upon a conceptual model of an *in planta* produced insecticidal dsRNA (Bachman et al., 2016), potential exposure to NTOs could occur through ingestion of the dsRNA containing tissues by herbivores and other soil dwelling organisms. Additionally, some plant material can move off-field into nearby surface waters and associated sediments as described in Carstens et al. (2012).

With topical application, it is possible to build upon the standard assumptions used for conventional pesticide sprays where soil is generally considered the primary receiving compartment in the environment with some off-site movement from spray (e.g. spray drift or soil run off) that may occur and could lead to NTO exposure in surface waters/sediments. For conventional pesticides, residue chemistry data are typically collected to provide the information necessary to determine the site, nature, and magnitude of residues in or on food/feed to estimate the exposure of the general population to pesticide residues and to set and enforce tolerances or maximum residue limits for pesticide residues in food/feed. For a topically applied dsRNA, the analysis of residues on plant tissues may provide additional data to inform the ERA as standard models for exposure of conventional sprayed pesticides (e.g. Kenaga nomogram) may overestimate the exposure of NTOs to sprayed dsRNA. For example, due to the barriers in plants to the uptake of sprayed dsRNA (e.g., cuticle, plant cell walls) the dsRNA applied to foliage would largely remain on the surface and be subject to environmental and microbially mediated degradation. As with conventional pesticides, the impact of product formulation such as stabilizing agents needs to be considered as part of the risk assessment, particularly if formulations are designed to overcome physical or biochemical barriers in target pests.

QUANTIFICATION OF dsRNA

The QuantiGene RNA assay has been used to accurately quantify dsRNA in environmental samples (Dubelman et al., 2014; Fischer et al., 2016; Albright et al., 2017; Fischer et al., 2017). This hybridization-based assay displays high specificity and can measure a single transcript from samples. It offers a high-throughput solution with repeatable results that have been accepted by regulatory agencies for product registration (U.S. EPA, 2017). Details on the use of QuantiGene can be found in Armstrong et al. (2013) with specifics on validation in soil matrices in Fischer et al. (2016). In side by side comparisons, QuantiGene results have been shown to be consistent with other methods for dsRNA detection such as northern blots, PCR, and UPLC (data not shown). The QuantiGene approach provides an advantage as it is more quantitative than a northern blot, less labor intensive, can quantify specific nucleic acid sequences unlike UPLC, and does not require amplification of the analyte as does PCR.

Parker et al. (2019) radiolabeled dsRNA with phosphorous-32 (^{32}P) and were able to quantify concentrations at the ng/g soil level. This approach allowed for refinement over previous work with QuantiGene by assessing dsRNA adsorption to soil particles and bio-degradation as part of the overall degradation characterization. Labeled dsRNAs were shown to degrade rapidly in soil suspensions, adsorb to particle surfaces, and be utilized by soil microorganisms. However, radiolabeling as an analytical method for nucleic acids has limits, as the labeled nucleotides are scavenged by organisms as a nutrient source, potentially confounding the degradation assessment and estimates of total recoverable radioactivity (TRR) would be a conservative over estimate of residues. From an ERA perspective, as with conventional pesticides, the bioavailability of active ingredients bound to soil particles is a consideration since long segments of dsRNA are negatively charged biopolymers that have the ability to bind to soil particles (Greaves and Wilson, 1969; Trevors, 1996; Draper, 2004; Pietramellara et al., 2009). Relatively harsh extraction methods are normally employed for conventional chemicals to free active ingredients from soil particles, but this approach is not likely to be suitable for dsRNA as it could destroy the test material. However, as discussed below dsRNA bound to soil particles is not likely to be a significant contributor to the ERA given the demonstrated rapid degradation of dsRNA in soil and soil suspensions, and the need for dsRNA to be unbound (and therefore subject to rapid degradation) to have any biologically meaningful activity.

FATE OF dsRNA IN SOIL, SURFACE WATERS, AND SEDIMENT

Laboratory microcosm studies enable robust replication and sampling to quantitatively assess degradation rates of dsRNA that can be used in risk assessments. A comprehensive series of environmental fate and degradation studies were performed in soil, surface water, and sediment for the insecticidal DvSnf7

dsRNA expressed in MON 87411 maize (Dubelman et al., 2014; Fischer et al., 2017). Results with DvSnf7 dsRNA are consistent with other published studies (Tabata et al., 1993; Zhu, 2006; Pietramellara et al., 2009; Eichmiller et al., 2016) that show nucleic acids are rapidly degraded in soil and aquatic environments. In these studies, a two-pronged approach was utilized employing both the QuantiGene assay and responsive insect bioassays to evaluate the environmental degradation of the dsRNA and the concurrent loss of functional bioactivity. This information was used to determine the potential exposure period for NTOs.

Dubelman et al. (2014) determined the biodegradation potential of the DvSnf7 dsRNA in three representative active agricultural soils with differing physicochemical characteristics. The estimated DT_{50} (time to 50% degradation) of the dsRNA in all soils was <30 hours and the DT_{90} (time to 90% degradation) values were <35 hours. These results combined with similar DT_{50} and DT_{90} values from insect bioassays demonstrating the loss of functional activity, indicate dsRNAs are unlikely to persist or accumulate in the soil, regardless of soil texture, pH, clay content, or other differences. In addition, Dubelman et al. (2014) demonstrated that the degradation kinetics of DvSnf7 dsRNA are independent of the initial dsRNA concentration as soil samples spiked with dsRNA at 0.3-37.5 µg/g soil displayed no apparent change in degradation kinetics.

Further work to elucidate the influence of dsRNA size, structure, and sequence on degradation kinetics was described in Fischer et al. (2016). The degradation of two dsRNA molecules with no significant shared sequence match and of different sequence lengths (968 and 100 bp) and structures (hairpin and linear) were evaluated in biologically active soil. The degradation kinetics of the two molecules were indistinguishable and displayed similar rapid degradation in soils as reported in Dubelman et al. (2014). These results suggest that unmodified dsRNAs are extremely labile and will not accumulate or persist in the environment. Joaquim et al. (2019) recently reported comparable results for DvSnf7 dsRNA degradation in tropical soils from Brazil.

DsRNAs have also been shown to degrade and not persist in aquatic systems, with half-lives of less than 3 days. Fischer et al. (2017) measured the degradation of DvSnf7 dsRNA in biologically active sediments and water collected from two separate natural systems representative of agricultural areas. The dsRNA was shown to rapidly degrade in the water phase of sediment-water microcosms. The dsRNA also degraded rapidly in a sediment-only system which lacked the overhead water column. As noted in Fischer et al. (2017), dsRNAs prepared in sterile (deionized) water appeared to be stable over the course of these studies, whereas the test systems utilized field collected and biologically active water and sediments indicating that the degradation of dsRNA is likely driven by microbial degradation. These results are consistent with previous work demonstrating that nucleic acids degrade rapidly and do not persist in aquatic compartments (Tabata et al., 1993; Zhu, 2006; Eichmiller et al., 2016).

To mimic the entry of a dsRNA into an aquatic system through either spray drift or transport by plant tissues, Albright et al. (2017) examined the dissipation of dsRNA within the water column and potential partitioning into the sediment compartment. As seen in Fischer et al. (2017), dissipation in the water column was rapid [< limit of detection (LOD) after 96 hours]. Non-significant levels of dsRNA were observed in sediment which the authors conclude may be due to rapid degradation in the water column precluding portioning into the sediment.

FATE OF dsRNA IN FOLIAR APPLICATIONS

There is a paucity of data describing the fate of foliarly-applied dsRNAs, and the data that are available is contradictory. Differences have been observed in the post application stability of the sprayed dsRNA product within controlled environments versus preliminary data from field environments and different detection/quantification methods have been employed that make comparison across studies difficult.

Mitter et al. (2017a; 2017b) reported that dsRNA suspensions sprayed on leaf surfaces under controlled conditions only offered 5 days of virus protection before degrading as confirmed by northern blot. Additionally, Cy3-labeled dsRNA applied to leaf surfaces and rinsed after 24 hours to mimic a rain event demonstrated that the dsRNA readily washed away as determined by confocal microscopy (Mitter et al., 2017a).

In a greenhouse experiment, San Miguel and Scott (2016) observed efficacy of up to 28 days for an insecticidal dsRNA applied to potato leaves. The dsRNA was not readily washed off once it had dried on the potato leaves. When the same dsRNA was incorporated into a gel and exposed to UV light for 1–2 hours, it was shown to be inactive. No quantification of the dsRNA used in these experiments was performed, but a responsive insect bioassay was used to determine the presence of active dsRNA for these studies.

In 2014 Bayer Crop Science conducted a pilot study to determine the magnitude and decline of residue levels of a topically applied 100 bp dsRNA on soybean under field conditions. The dsRNA was the same 100 bp sequence as used in Fischer et al. (2016) and displayed rapid dissipation in soil. The study was conducted under procedures consistent with Good Laboratory Practices (GLP).

The study site was in Puerto Rico and the soybean was produced under agronomic conditions and practices typical in that region. The study consisted of a single untreated control plot (treatment 1) and two treatment plots (treatments 2 and 3) with two replicates each (**Table 1**). In treatment 2, dsRNA was applied at target rate of 59.3 g ai/ha at three separate applications: V4/R1, V10/R3, and 7 days before harvest whereas treatment 3 omitted the 7 day preharvest treatment. Each plot consisted of four rows planted on 0.76 m rows that were 15.2 m long (approximately 46.5 m^2 plot area). The seed used was a commercial variety of RoundUp Ready/Insect Protected soybean (Asgrow). Applications were made with a backpack CO_2 sprayer with a flat fan nozzle. Weather during the study was similar to the historical average (mean temperature 22.1–30.7°C; 9.5 cm mean

rainfall) and no rain events were recorded during the spraying or whole plant residue collection period. Irrigation was provided *via* drip tape. Aerial portions (above soil) of the plants were collected to determine residue levels of the dsRNA *via* the Quantigene assay. Whole plant samples were immediately frozen on dry ice and maintained frozen on dry ice or at −80°C until analysis. No growth or developmental abnormalities were observed during the field study.

Contrary to results reported from similar experiments in controlled environments, under field conditions the concentration of the foliarly-applied dsRNA rapidly declined with a ~95% reduction 3 days after treatment (DAT) and an almost 99% reduction 7 DAT. The estimated dissipation kinetics provide a DT_{50} of 0.7 days and a DT_{90} of 2.3 days for treatment 2 and a DT_{50} of 0.5 days and DT_{90} of 1.9 days for treatment 3 (**Figure 1**). Additionally, negligible amounts (0.19 ng/g fw) of dsRNA were detectable in harvested grain from the soy plants at maturity in treatment 2, which included an application of dsRNA 7 days prior to grain harvest. No detectable residues of the dsRNA in grain was found in treatment 3 which lacked the 7 day preharvest treatment, thus supporting the conclusion that dissipation of the foliarly applied dsRNA was due to decline of residues on or near the plant surface and not due to uptake and degradation within the plant vascular system. This conclusion is supported by a similar GLP field study (not reported) performed with potatoes in three locations in the United States (Iowa, Wisconsin, and Washington) where two applications of dsRNA at 59.3 g ai/ha (4–5 weeks after planting and 28 days after the initial application) of the same 100 bp dsRNA and a 397 bp dsRNA with activity against Colorado Potato Beetle did not result in detectable residues in potato tubers.

Several reasons may exist for the observed instability post application including photodegradation, wash-off due to rain or dew, and microbial degradation. UV light is known to degrade nucleic acids (Kundu et al., 2004) and San Miguel and Scott (2016) observed that dsRNA lost biological activity after exposure to UV light. Contrasting results were shown by San Miguel and Scott (2016) and Mitter et al. (2017a; 2017b) in terms of stability of sprayed dsRNA after washing, however no rainfall was recorded during the 7 DAT in the Bayer study. The rapid degradation of topically applied dsRNA in field versus controlled environments is not unexpected given the lability of nucleic acids in the environment and rapid degradation in the presence of microbes (Pietramellara et al., 2009; Parker et al., 2019).

Strategies to mitigate degradation could come from the formulation of end products such as addition of UV protectants, rain-fastness agents, and/or antimicrobials or physical encapsulations to limit microbial activity. In an environmental study in which dsRNA was protected by formulation ingredients by incorporation of dsRNA into

TABLE 1 | Summary of pilot field study to evaluate stability of topically applied dsRNA on soybean plants using the QuantiGene assay.

Treatment	Application rates (target) and timing	Spray rate (liters per hectare)	Carrier	Sampling	Residue level average concentration[4] ng/g fw (min.–max.)
1 (Control, 1 plot)	Untreated	187	Ultrapure water plus Silwet L-77 at	12 whole plants; plus 1 kg grain at maturity	N/A
2 (2 replicate plots)	59.3 g ai/ha[1] at three separate applications: V4/R1, V10/R3, and 7 days before harvest	187	0.5% v/v	12 whole plants per plot collected and pooled for analysis at 0,3, and 7 DAT[2]; plus 1 kg grain at maturity	0 DAT[2]: 4166 (4158-4174) 3 DAT[2]: 242 (235-249) 7 DAT[2]: 66.23 (56.01-76.44) Seeds: 0.19 (0.15-0.23)
3 (2 replicate plots)	59.3 g ai/ha[1] at two separate applications: V4/R1, and V10/R3	187		12 whole plants per plot collected and pooled for analysis at 0,3, and 7 DAT[3]; plus 1 kg grain at maturity	0 DAT[3]: 1317 (1014-1619) 3 DAT[3]: 50.33 (50.17-50.49) 7 DAT[3]: 25.66 (25.47-25.85) Grain: Not Detected (< LOD)

[1]For treatment 2 actual applied rates were 54.9–63.9 g ai/ha. For treatment 3 actual applied rates were 57.2–59.4 g ai/ha.
[2]Days after treatment (DAT) refers to time point following initial application at V4/R1.
[3]Days after treatment (DAT) refers to time point following second application at V10/R3.
[4]The average residue concentration was calculated at each sampling interval for two replicate plots for treatments 2 and 3. dsRNA concentrations for each replicate plot provided in parenthesis.
Assay's LOD, 0.0015 ng/g fw; Assay's LOQ, 0.0180 ng/g fw; fw, fresh weight; N/A, not applicable.

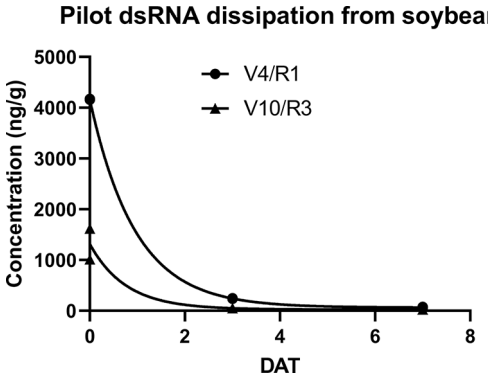

FIGURE 1 | dsRNA dissipation in soybean for treatments 2 (labeled V4/R1) and treatment 3 (labeled V10/R3) from pilot study. Treatment 2 measurements occurred at 0, 3, and 7 DATs of initial application at V4/R1. Treatment 3 measurements occurred at 0,3, and 7 DATs of second application at V10/R3. Estimated dissipation rate kinetics for V4/R1 are: DT_{50} of 0.7 days and DT_{90} of 2.3 days. Estimated dissipation rate kinetics for V10/R3 are: DT_{50} of 0.5 days and DT_{90} of 1.9 days. Dissipation curves and estimates calculated from plotted individual replicates using a first order exponential decay model in Prism GraphPad v8.2.0. No error bars are illustrated as individual replicates are shown.

layered double hydroxide (LDH) nanosheets known as "BioClay", virus protection of dsRNA applied to tobacco leaf surfaces was increased and extended from 5 to 20 days (Mitter et al., 2017a). Whitfield et al. (2018) demonstrated that cationic polymers applied to soil affect degradation kinetics and increase the lifetime of dsRNA in soil. Persistence of dsRNA in soil of up to 3 weeks was achieved through the application of a shaped poly(2-(dimethylamino) ethyl acrylate) analog. Given that these studies were done in protected environments, information is not yet available as to how these formulations will directly or indirectly impact NTOs or exposure scenarios for the dsRNAs contained in them.

DISCUSSION

To enable effective ERAs for dsRNA crop protection products, the dsRNA must be successfully measured in relevant environmental compartments based on intended use patterns. For topically applied dsRNA products, the primary environmental compartments are treated plants, soil, and secondarily surface waters/sediment. The QuantiGene assay is an appropriate and efficient analytical method for determining the environmental fate of dsRNA agricultural products and has been used successfully in registration applications for transgenic plants expressing insecticidal dsRNAs. Additional methodologies such as radiolabeling dsRNA offer potential refinements to the exposure assessment and may be useful to answer questions regarding the binding of dsRNA to soil particles versus degradation due to the potential confounding use of labeled nucleotides as a nutrient source. This technique should only be used as part of problem formulation for a given product or use pattern if further refinement of the exposure scenario is required. Standardization of analytical methods for quantification of dsRNA in environmental matrices will enhance the reconstructability, repeatability, and comparison of these types of studies and provide benefits to the regulatory process for dsRNA product approval.

Results from numerous environmental fate studies with unformulated dsRNAs demonstrate a high lability of these molecules and low potential for persistence in the environment including soil, sediment, and surface water compartments. Microbial degradation of nucleic acids in environmental compartments has been shown to be a key driver for this rapid degradation and lack of persistence. Preliminary results suggest that foliarly-applied dsRNA is subject to rapid degradation under field conditions. For these dsRNA products, more data are needed to understand the drivers of stability on leaf surfaces especially under field conditions as low environmental stability could affect product efficacy. Modifications to dsRNA or formulations that alter stability in the environment, or overcome physical or biochemical barriers in target pests, may require additional studies to determine their effects on dissipation and degradation rates and any potential increase in exposure to relevant NTOs.

Formulations or other approaches to mitigate environmental degradation may lead to more successful products but may change the known degradations kinetics of dsRNAs. The formulation of these products and the resultant impacts on the stability of the dsRNA in environmental compartments will need to be addressed in problem formulation on a case by case basis to ensure an efficient risk assessment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PB, JF, and GW conceptualized the content of the manuscript. PB wrote the first draft of the manuscript. EU-W and GW designed the pilot studies. ZS developed and validated the residue analytical method and conducted the residue analysis. All authors contributed to the manuscript revision, read, and approved the submitted version.

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A Perspective on RNAi-Based Biopesticides

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Sustainable agriculture relies on practices and technologies that combine effectiveness with a minimal environmental footprint. RNA interference (RNAi), a eukaryotic process in which transcript expression is reduced in a sequence-specific manner, can be co-opted for the control of plant pests and pathogens in a topical application system. Double-stranded RNA (dsRNA), the key trigger molecule of RNAi, has been shown to provide protection without the need for integration of dsRNA-expressing constructs as transgenes. Consequently, development of RNA-based biopesticides is gaining momentum as a narrow-spectrum alternative to chemical-based control measures, with pests and pathogens targeted with accuracy and specificity. Limitations for a commercially viable product to overcome include stable delivery of the topically applied dsRNA and extension of the duration of protection. In addition to the research focus on delivery of dsRNA, development of regulatory frameworks, risk identification, and establishing avoidance and mitigation strategies is key to widespread deployment of topical RNAi technologies. Once in place, these measures will provide the crop protection industry with the certainty necessary to expend resources on the development of innovative dsRNA-based products. Readily evident risks to human health appear minimal, with multiple barriers to uptake and a long history of consumption of dsRNA from plant material. Unintended impacts to the environment are expected to be most apparent in species closely related to the target. Holistic design practices, which incorporate bioinformatics-based dsRNA selection along with experimental testing, represent important techniques for elimination of adverse impacts.

Keywords: RNAi, biopesticide, topical application, dsRNA, crop protection

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BACKGROUND

The demands on global agriculture are expected to escalate in the coming decades, with the population likely to increase to ~9 billion by 2050 (Organisation for Economic Co-operation and Development Staff, and Organisation for Economic Co-operation and Development., OECD environmental outlook to 2050, OECD environmental outlook,OECD, Paris, 2012). Many additional factors are expected to exacerbate the challenges faced by global agriculture, including a move toward greater consumption of more nutritious foods in developing countries with improving economies, decreases in arable land due to urban expansion and land degradation, and perhaps most importantly, adverse effects generated by climate change (FAO, 2009). Climate

change associated impacts could include reduced yields due to greater temperatures and extreme weather events, and increased losses owing to expanded and changing ranges of crop pests and pathogens (Chakraborty and Newton, 2011; IPCC, 2014).

Accordingly, sustainable yield increases in the face of the global constraints to production are a necessity. One area where significant productivity gains can be made is limiting crop losses associated with pests and pathogens. Currently, resistant cultivars, chemical pesticides, and integrated management practices are the most efficient methods to respond to biotic challenges. However, since the latter half of the last century, concerns have grown about the use of chemical pesticides, in particular their impacts on human and environmental health, including the lack of differentiation of targets and non-target organisms in their mode-of-action, and widespread development of pesticide resistance. Consequently, the development of innovative and environmentally sustainable approaches to crop protection has become increasingly important.

Among the most notable paradigm shifts in agriculture over the past 50 years is the commercial deployment of genetically modified organisms (GMOs). Crops such as cotton, maize, and soybean have been engineered to be resistant to specific pests and pathogens, yielding staple commodities in many markets. Significant barriers to the uptake of GMOs have however been community acceptance, the cost and time involved, obtaining regulatory approval, and the lack of transformation protocols for many crop species. Herein, we provide a perspective on the development, limitations and risks associated with non-GMO dsRNA-based products which aim to use RNAi to provide protection from crop pests and pathogens in a highly-targeted manner without the need for plant genetic modification.

THE FUNCTIONAL BASIS OF DSRNA-BASED PRODUCTS: RNAi

RNAi comprises a conserved set of mechanisms that eukaryotes use for regulating RNA transcript abundance. The physiological consequences of RNAi were first identified almost a century ago by Wingard, who observed in tobacco that Tobacco Ringspot Virus infection in the lower leaves was associated with resistance to secondary infection in the upper leaves (Wingard, 1928). The advent of plant and fungi genetic modification lead to observations that the integration of transgenes homologous to endogenous genes sometimes resulted in the suppressed expression of both, a process termed "co-suppression" in plants (Napoli et al., 1990) and "quelling" in fungi (Romano and Macino, 1992). Subsequently, the defensive nature of the process was demonstrated by co-suppressed of viral transcripts, with a transgene expressing a portion of the Tobacco Etch Virus coat protein (CP) able to induce delayed resistance to the virus sequence-specific manner (Lindbo et al., 1993). Fire et al. established that in the nematode *Caenorhabditis elegans*, double-stranded RNA (dsRNA) was a far more potent suppressor of target transcript expression than single-stranded RNA (ssRNA) (Fire et al., 1998). This discovery, for which Fire and co-author Mello were awarded the Nobel Prize, marked the birth of the RNAi revolution.

Practical uses of RNAi were rapidly developed, with transgene-expressed dsRNA employed to induce virus resistance and gene silencing in plants (Waterhouse et al., 1998). Over the following years, other components of the pathway were elucidated. Intermediaries in the form of small ~25 nt antisense RNAs were identified as guides for target RNA degradation (Hamilton and Baulcombe, 1999; Zamore et al., 2000). Dalmay and co-workers showed that the RNA-dependent RNA polymerase RDR6 was recruited to generate dsRNA from target transcripts in plants, leading to a feedback loop of increased small interfering RNA (siRNA) abundance and silencing potential (Dalmay et al., 2000). This process is also evident in fungi and nematodes (Baulcombe, 2004). Other fundamental components then identified included the RNase III domain-containing enzyme responsible for dsRNA cleavage in *Drosophila*, which was termed "Dicer" (Bernstein et al., 2001). Dicer-like genes were also evident in plants and fungi (Jacobsen et al., 1999; Schauer et al., 2002). Members of the conserved Argonaute family were recognized as components of the RNA-induced silencing complex (RISC), which mediated cleavage of the target transcript (Liu et al., 2004; Baumberger and Baulcombe, 2005). Thus, the primary constituents of the RNAi pathway had been identified, with application of the mechanism rapidly advancing across biological fields.

From a risk analysis perspective, the elucidation of many components of the RNAi pathway had important implications for pest and pathogen control applications. RNAi was demonstrated to be highly sequence-specific, allowing for concise dsRNA-directed targeting of transcripts for degradation, however the conserved nature of the pathway among eukaryotes entailed that unintended impacts on non-target organisms (NTOs) could be evident in the presence of the dsRNA if sufficient transcript homology existed.

RNAI FOR PROTECTION AGAINST PLANT PESTS AND PATHOGENS

An important factor that advanced RNAi as a crop protection measure was the observation that the plant's response to virus incursion was functionally related to the response to transgenes [e.g., (Ratcliff et al., 1997; Ruiz et al., 1998)]. The non-cell autonomous nature of RNAi in plants, with local and long distance systemic movement of silencing signals, indicated that co-option of the pathway could provide highly-selective systemic resistance (Tenllado et al., 2004). Though plants had previously been transformed with single-stranded constructs to induce virus resistance, the advantages of expressing dsRNA became clear as the components of the RNAi pathway were characterised. Using a hairpin construct, Wang et al. generated complete resistance to Barley yellow dwarf virus-PAV in transgenic barley,

demonstrating the efficiency of the technique in an important commodity crop (Wang et al., 2000).

Though RNAi had previously been used as a tool for examining gene function in insects (Belles, 2010), Baum et al. developed an orally-applied (via artificial diet or transgenic maize) RNAi approach for inducing mortality in the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) *via* targeting various V-ATPase subunits, along with α-tubulin (Baum et al., 2007). In the same year, Mao et al. reported the impairment of growth of cotton bollworm (*Helicoverpa armigera*) by feeding plant leaf material expressing a dsRNA specific to a cytochrome P450 gene (Mao et al., 2007). The approval of the first commercial GMO varieties expressing a dsRNA against an insect pest would not occur until 2017, with Monsanto and Dow's SMARTSTAX PRO maize incorporating a dsRNA against another western corn rootworm gene, *Snf7* (Head et al., 2017). At around the same time, approval was granted for apple and potato expressing dsRNAs for regulation of endogenous gene expression for quality enhancement (Waltz, 2015; Baranski et al., 2019).

In addition to viruses and insects, RNAi has also been adopted for control of many other plant pests and pathogens in a research setting, including bacteria such as *Agrobacterium*, fungi such as powdery mildew, and nematodes such as Root knot nematodes (Rosa et al., 2018). Limitations to the genetic modification approach to crop protection have however been readily apparent for some time, and include low public acceptance in many markets and the inability to genetically transform many crop species (Zotti et al., 2018). Accordingly, much of the recent focus on RNAi for crop protection has shifted toward non-transformative strategies (Dalakouras et al., 2019).

DEVELOPMENT OF TOPICALLY-APPLIED RNAi

Functional foliar application of dsRNAs targeting the plant viruses Pepper mild mottle virus (PMMoV), Alfalfa mosaic virus (AMV) and Tobacco etch virus (TEV) was first reported by Tenllado and co-workers in 2001 (Tenllado and Diaz-Ruiz, 2001). In a statement that was to prove prescient, the authors noted that topical application of *in vitro*-expressed dsRNA for protection against plant viruses could be commercially viable provided dsRNA production became inexpensive and an adequate means of delivery was developed (Tenllado and Diaz-Ruiz, 2001). The same authors attempted to reduce the costs of the dsRNA by applying a crude extract of *E. coli* HT115 expressing the same dsRNA fragments used previously, and achieved similarly positive results with viral co-inoculation, though the window of resistance was limited to five to seven days (Tenllado et al., 2003). Additional risks of such an approach relative to the application of purified dsRNA are however evident. These include the potential for toxic fermentation by-products, the presence of selective antibiotics used in growth media, and the uncertain GMO-status of a non-purified product.

Following Tenllado and co-workers' pioneering work, a limited number of reports were evident over the proceeding decade. Gan and co-workers generated dsRNA for topical application against the Sugarcane mosaic virus coat protein using the HT115 system developed earlier by Tenllado (Gan et al., 2010). Lau et al. also used bacterial extracts to generate dsRNA against Cymbidium mosaic virus coat protein (Lau et al., 2014). In more recent years protection from many plant viruses across multiple families has been successfully provided by topical application of dsRNA (Mitter et al., 2017a).

Given the devastation caused by fungal pathogens to crop yield worldwide, the successful topical application of dsRNA to control a fungal infection was significant. Koch et al. showed that *Fusarium graminearum* growth could be inhibited by direct application on detached barley leaves of a dsRNA targeting three CYP450 genes (Koch et al., 2016). Importantly, the ability to inhibit fungal growth spread systemically in the leaf, controlling the pathogen in unsprayed areas. In a recent publication, Höfle et al. demonstrated the length of the sprayed dsRNA impacts on the effectiveness of individual *F. graminearum* CYP gene knockdown, with >1,500bp constructs being much less effective than 200–500 bp constructs (Höfle et al., 2019). By targeting two Dicer-like genes in *Botrytis cinerea*, Wang and co-workers effectively controlled the pathogen on fruit, vegetable and flower surfaces, demonstrating that RNAi could play a role in the post-harvest protection of agricultural produce in addition to pre-harvest protection (Wang et al., 2016). McLoughlin et al. were also able to decrease fungal infection and reduce symptoms in *B. cinerea*, as well as *Sclerotinia sclerotiorum*, *via* foliar application of dsRNA on *Arabidopsis* and *Brassica napus* leaves (McLoughlin et al., 2018).

Relative to viruses and fungi, the development of topical RNAi strategies for protection against arthropod pests has been technically demanding for a range of reasons, including a lack of amplification of the RNAi silencing signal and dsRNA degradation during ingestion (Niu et al., 2018). While oral uptake of dsRNAs targeting critical genes had been shown to induce mortality in some arthropods, transferring delivery from an artificial diet to a topical application strategy has proven difficult. When arthropod pests take up dsRNAs/siRNAs from the plant surface or from internal tissues such as vascular bundles, the abundance of dsRNAs/siRNAs transported to cells where they are effective is comparatively low without protective and uptake enhancement measures being put in place (Niu et al., 2018). Additionally, a study by Biedenkopf et al. indicates that the abundance of RNAi effectors and their ability to induce silencing decreases with distance from the site of exogenous application (Biedenkopf et al., 2019). Interestingly, the authors noted that in the case of barley, the topically-applied dsRNA enters the plant and spreads systemically to leaves, shoots and roots *via* the phloem. It was also evident that the internalised dsRNA was at least partially processed into siRNAs, which could also be detected in distal tissues. These technical barriers have however proven surmountable in some circumstances. The coleopteran Colorado potato beetle was recognised to be highly susceptible to foliar-applied dsRNA, as demonstrated by San Miguel and Scott on potato leaves (San Miguel and Scott, 2016). Non-foliar application methods have also proven successful, with root uptake of target-specific

dsRNAs generating mortality in brown planthoppers and Asian corn borers (Li et al., 2015).

Due to circumvention of genetic modification of the host crop, major impediments to adoption of effective topical RNAi approaches are being addressed, including negative public perception of GM-based produce and the inability to transform many important agricultural species. Current research and development of topically-applied RNAi technologies typically focuses on two themes: selecting mortality-maximising target genes specific to a given species, and ensuring topically-applied dsRNAs are sufficiently stable for an optimum protection window. Bioinformatics-based approaches have been used extensively for off-target impact mitigation during the design phase and are discussed below. Stabilization of the dsRNA is a multifaceted issue that is characteristically dependent on the application scenario. Degradation of dsRNA in the environment can occur *via* the actions of ribonucleases and/or UV radiation, both of which are ubiquitous in agricultural settings. The stability of an arthropod-targeting dsRNA should also be sufficient for ingestion, necessitating persistence in non-neutral pH gut conditions prior to delivery to relevant tissues. The use of nanocarriers as components of the delivery system is an option to surmount these hurdles. Nanomaterials have dimensions of less than 100 nm resulting in high surface area to volume ratios, and can be engineered with both protective and slow-release properties for their payloads (Ghormade et al., 2011). Here we present a case study on delivery of dsRNA using clay nanoparticles as carriers, aimed at addressing some of the issues associated with naked dsRNA applications.

CASE STUDY – BIOCLAY FOR PROTECTION AND SLOW RELEASE OF DSRNA ON PLANT SURFACES

Pioneering work by Tenllado and co-workers on topically-applied RNAi identified the short window of protection offered by a foliar application as an impediment to widespread adoption of the technology (Tenllado and Diaz-Ruiz, 2001). This has also been identified as a key factor in various publications emerging in the last decade on topical application of RNAi [e.g., (Yu et al., 2013; Zotti et al., 2018)].

Mitter et al. explored the use of dsRNA complexed with layered double hydroxide (LDH) nanosheets, termed BioClay, as a spray application (**Figure 1**) (Mitter et al., 2017b). Employing BioClay allowed the window of protection from viral pathogens to be expanded to 20 or more days. Importantly, LDH itself is biocompatible and used in human therapeutics (Del Hoyo, 2007; Kuthati et al., 2015). LDH also safely degrades in the presence of mildly acidic conditions, thus minimising risk of excessive persistence of the dsRNA in the environment. Abating risk while maintaining effectiveness will require similarly novel solutions during the conception of many RNAi-based products, indicating the benefits of risk identification at the earliest stages of development.

MITIGATION AND AVOIDANCE OF RISKS ASSOCIATED WITH RNAi-BASED PRODUCTS

All technologies, whether currently in use or novel, carry a set of risks, which can be either avoided or mitigated through identification, careful planning and design, and safe use practices. Whilst RNAi-based technologies offer clear and obvious safety benefits relative to many existing crop protection products, an analysis of risk is still key to their deployment. The generalized risks associated with environmental application of dsRNA fall into two areas: unintended impacts on human health and unintended impacts to the broader environment (**Figure 2**). When combined with other agents in a formulation, risk analysis of the dsRNA component is less generalizable and should be examined on a case-by-case basis.

RISKS TO HUMAN HEALTH

There are three primary routes to human exposure for topically applied dsRNA: ingestion, inhalation, and dermal. For dsRNA to induce a cellular response in humans, it would need to pass through multiple and redundant barriers irrespective of the exposure route. Putative impacts of dsRNA that is internalized could be sequence independent and/or sequence specific. Humans possess an innate immune system that recognises dsRNA in a non-sequence specific manner *via* multiple receptors (DeWitte-Orr et al., 2009; Whitehead et al., 2011). Similar to the situation in other higher eukaryotes, in humans dsRNA can be recognized as a pathogenic signature by the cell, which can then produce an interferon response. Notably, generating such a response *via* inhalation or ingestion is challenging. Humans have a long history of dietary consumption of considerable amounts of dsRNA from virus-infected plant material without any indication of detectable effects, likely due to the rapid degradation of nucleic acids in the stomach in the first instance (Jensen et al., 2013). Few studies have been carried out on the non-sequence specific impacts of dsRNA *via* the inhalation route, as the synthetic dsRNA analogue polyinosinic:polycytidylic acid (poly I:C), which is notable for its ability to induce inflammation and a hypersensitive response, is generally used in mouse and cell line studies as a dsRNA substitute [e.g., (Mahmutovic-Persson et al., 2014)]. The application of poly(I:C) *via* injection under mouse wound scabs has also been shown to induce retinoic acid synthesis and hair follicle regeneration (Kim et al., 2019), though such a route to human exposure would not be considered common occurrence for an RNAi-based biopesticide.

For dsRNA to pose a significant risk to human health in a sequence-specific manner is considered less likely, as a dsRNA would need to be translocated inside a cell rather than to receptors on its surface. The inability to successfully develop RNAi-based therapies indicates the magnitude of the delivery problem (Chen et al., 2018). Instability of non-coding RNAs in biological fluids due to the abundance of endogenous nucleases, and subsequent removal *via* the kidneys, along with inabilities to cross vascular and cellular barriers are cited as key constraints (Chen et al., 2018). Environmental dsRNA without specific

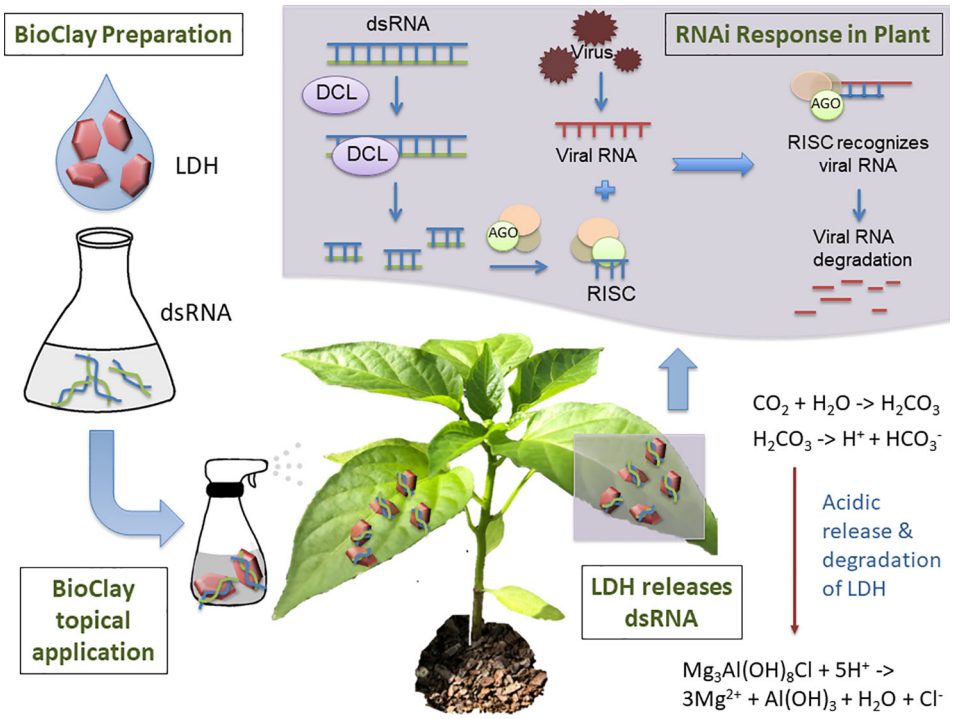

FIGURE 1 | Topical application of BioClay allows for extended RNAi-mediated protection from plant viruses. BioClay is a complex of double-stranded RNA (dsRNA) and layered double hydroxide (LDH). BioClay is prepared by mixing dsRNA and LDH in solution and is applied as a foliar spray. Moisture and carbon dioxide combine to allow acid release of the dsRNA, with LDH degrading to its constituents. The dsRNA can subsequently be taken up by the plant and prime its RNA machinery to degrade homologous viral RNAs. Due to the stabilization and slow release of dsRNA, resistance to the target virus relative to naked dsRNA can be extended from days to weeks.

protective measures would be similarly affected. An additional constraint for introduced dsRNAs or dsRNA-derived siRNAs is that these sequences must have sufficient homology to endogenous transcripts to induce transcript degradation. Even when this homology is evident, several studies have shown that any impact is likely to be negligible, likely due to the aforementioned delivery constraints. Petrick and co-workers conducted 28-day toxicology trials using dsRNA and siRNAs on mouse models (Petrick et al., 2015). Even with abundant consumption of dsRNAs and siRNAs completely homologous to the mouse vATPase gene, no suppression of gene expression nor any physiological impacts were evident. Indeed, consumption of plant material containing dsRNA capable of generating siRNA homologous to human transcripts is a further indication that sequence-specific impacts are likely unwarranted, at least in the ingestion pathway (Jensen et al., 2013). As noted by Food Standards Australia New Zealand in relation to consumption of dsRNAs from GMOs, "There is no scientific basis for suggesting that small dsRNAs present in some GM foods have different properties or pose a greater risk than those already naturally abundant in conventional foods" (FSANZ, 2013). Formulation of dsRNA-based products with other constituents could however impact human exposure pathways and may require assessment on a case-by-case basis.

RISKS TO THE ENVIRONMENT

Unintended environmental consequences of RNAi-based products are often case-specific. For example, a beneficial non-target insect that is closely related to a targeted insect pest may be similarly susceptible to environmental RNAi. If there were sufficient dsRNA sequence homology to a key gene, and the beneficial insect had a similar range and feeding patterns, comparable effects would be predicted for mortality. An example of such non-target impacts on related insect species is demonstrated by Baum et al., (2007). Four coleopteran species were fed dsRNA designed to induce mortality. The target Western corn rootworm (WCR) along with the Southern corn rootworm displayed significant mortality upon consumption of a WCR *V-ATPase A*-targeting dsRNA. Colorado potato beetles (CBP) also displayed significant mortality, but the dsRNA was less effective than one directly targeting the CPB *V-ATPase A*. Lastly, the cotton boll weevil suffered no mortality, even with a dsRNA targeting the endogenous CWV *V-ATPase A*. Susceptibility to dsRNA can vary between species, making accurate prediction of gene knockdown a complex issue. In insects, coleopteran species are generally considered the most susceptible to RNAi, with dipterans and hymenopterans sometimes susceptible, and lepidopterans and hemipterans

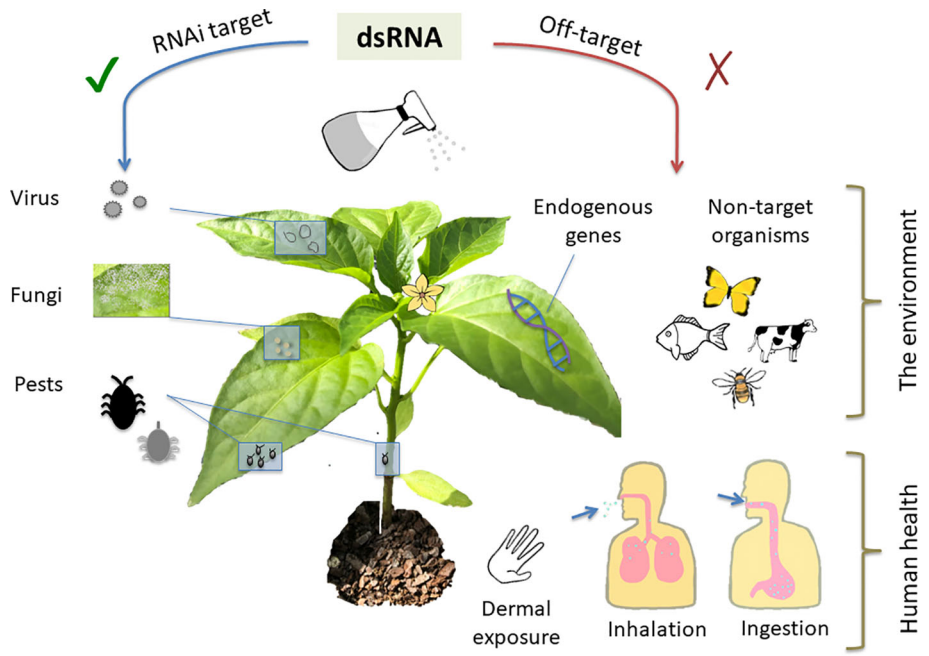

FIGURE 2 | As a crop protection measure, topically-applied dsRNA should be effective against specific pests and pathogens while avoiding unintended adverse consequences. Topically applied double-stranded RNA (dsRNA) can be used to generate resistance to pathogenic viruses and fungi, and pests such as insects. Off-target impacts to be avoided include silencing of crucial host plant and other non-target organism genes. Potential routes of exposure to humans including operators applying the dsRNA along with consumers of treated products could occur *via* dermal exposure, inhalation, and ingestion.

rarely susceptible (Baum and Roberts, 2014; Christiaens et al., 2018).

The development of crop plants expressing dsRNAs against insect pests has led to a number of studies assessing the risks associated with their deployment. Vélez et al. examined the impact on honey bees (*Apis mellifera* L) of maize pollen expressing a dsRNA targeting *V-ATPase A* transcripts of either the target WCR, or the same transcript in the bee itself (Velez et al., 2016). There were no impacts on survival evident at larval or adult stages by either dsRNA, indicating honey bees are not readily susceptible to environmental dsRNA, even with complete sequence homology. Similarly, Tan et al. tested a dsRNA directed against the WCR *DvSnf7* transcript (Tan et al., 2016). Even at higher concentrations than would be present in the field, no impact was evident on honey bee larvae or adults. In a comparable approach to Vélez et al, Pan and co-workers assessed the impact of WCR and endogenous *V-ATPase A* dsRNAs on monarch butterfly (*Danaus plexippus* L) neonates (Pan et al., 2017). No impact on target gene expression or survivability was evident. As with the honey bee, the monarch butterfly did not appear to be susceptible to environmental RNAi. Pan and co-workers also investigated the potential risks of environmental RNAi to the slender springtail (*Sinella curviseta*), again using WCR and endogenous *V-ATPase A* targeting dsRNAs (Pan et al., 2016). Based on artificial diet assays, the authors concluded that adverse impacts to the soil-borne arthropod were negligible. Haller et al. used a WCR *V-ATPase A* dsRNA to determine the responses of two coleopteran ladybird species (*Adalia bipunctata* and *Coccinella septempunctata*) (Haller et al., 2019). As with other coleopteran species, the ladybird species were sensitive to diet-applied dsRNAs, though administered concentrations were much greater than were expected in field conditions. Notably, the degree of negative impacts was associated with the number of homologous matching 21nt sub-sequences for both species, with six matching the *A. bipunctata* transcript and 34 matching the *C. septempunctata* mRNA.

As target sequences become less conserved, the likelihood of inducing deleterious off-target effects is reduced, owing to an inability to produce sufficient off-target homologous siRNAs. Though genetically divergent from the target, one particular off-target organism that is always likely to come into contact with an applied dsRNA is the crop itself. The effectiveness of foliar-applied dsRNA against plant viruses indicates at least a portion of the dsRNA pool is internalized by the plant, which then primes the host RNAi system against viral RNAs. Techniques such as parallel analysis of RNA ends (PARE) have been proposed for identification of endogenous mRNA targets in dsRNA-expressing plants (Casacuberta et al., 2015). Such molecular techniques may also be of use in detecting off-target impacts on crop species following application of RNAi-based biopesticides.

To counter unintended impacts on closely related beneficial species, and indeed any other non-target species the dsRNA may come into contact with, an understanding of the setting in which the RNAi technology will be applied is key, along with careful target sequence selection and subsequent bioinformatics-based design.

BIOINFORMATICS FOR IDENTIFICATION AND AMELIORATION OF OFF-TARGET IMPACTS

Degradation of transcripts by the RNAi machinery is directed by siRNAs of ~21–22nt in length, which are generated *via* Dicer from the applied dsRNA. The pool of all possible sense and antisense siRNA sequences derived from a dsRNA can be computationally calculated, allowing for simple identification of homologous non-target transcripts. The degree of homology required to efficiently induce expression knockdown varies, and remains an area of ongoing study. For example, insect feeding studies have shown one or more 19 nucleotide matches between a dsRNA and transcript can reduce transcript expression, which could have deleterious effects if environmental dsRNA were sufficiently abundant (Christiaens et al., 2018).

Taking a precautionary approach to dsRNA design, regions of target genes can be selected to ensure homology to off-target transcripts is minimised, and any contiguous matches above a set limit are identified and avoided (Naito et al., 2005). OfftargetFinder, a web application developed by Good and associates, serves as an example of this approach (Good et al., 2016). Using a database of arthropods and other key species, the software indicates which off-target species have 21nt matches, and allows for the operator to test different regions of a target gene to minimise off-target hits. This application has been used to examine CBP and WCR target genes (*β-actin* and *DvSnf7* respectively) for putative off-target impacts on the lady beetle *Coleomegilla macula* and the red flour beetle *Tribolium castaneum* (Allen, 2017). Another common approach to identify off-target hits has been to use the BLAST search tool. For example, Ulrich et al. employed BLAST to identify contiguous matching regions of 17nt or more between selected off-target insect species and RNAi target genes identified in a large-scale screen of *T. castaneum* (Ulrich et al., 2015).

There are two caveats to the use of the aforementioned approaches, preventing bioinformatics-based selection being the sole arbiter of unintended impacts of a dsRNA. Firstly, an off-target species that possesses a transcript with homology above an arbitrary level may be unaffected for a multitude of reasons; the dsRNA may not be taken up or it may not be transported to a cell where the off-target transcript is expressed (as is likely the case with mammals), transcript degradation may have no impact due to redundancy or other factors, all of which result in no identifiable physiological impact. Consequently, bioinformatics analyses based on homology alone are likely to vastly overestimate the likelihood of off-target impacts, particularly given the abundance of each discrete siRNA generated from a dsRNA is low.

The second deficiency of bioinformatics-based analyses is the lack of genome and transcriptome information currently available for certain beneficial and non-pest species. Fortunately, as genome sequencing costs are rapidly reducing, the public availability of new sequence data that can inform such analyses continues to grow. Focused sequencing may however be required to fill knowledge gaps in specific circumstances.

Notwithstanding these caveats, it is clear a holistic approach to risk avoidance and mitigation has bioinformatics-based design as a component, but is also strongly informed by biological data and an understanding of the biological and ecological systems in which the dsRNA will be deployed.

CASE STUDY: REGULATORY ENVIRONMENT IN AUSTRALIA PERTAINING TO DSRNA-BASED PRODUCTS FOR TOPICAL APPLICATION TO PLANTS

A critical step in bringing innovative products to market is dialogue between developers and the regulatory authority. In addition to ensuring community and environmental safety, this action provides certainty to developers. Here we provide a case study on the Australian regulators' analysis of where topically-applied RNAi products fit within the existing legal landscape.

Prior to 8th October 2019, topically applied RNAi-based products in Australia were regulated by the Office of the Gene Technology Regulator (OGTR) and the Australian Pesticides and Veterinary Medicines Authority (APVMA). However, this situation changed on 8th October 2019 when approved amendments to the *Gene Technology Regulations 2001* come into effect. The OGTR's *Technical Review of the Gene Technology Regulations 2001* clarifies the regulatory status of organisms developed using a range of new technologies and ensures that new technologies are regulated in a manner commensurate with the risk they pose. In the case of RNA-induced gene silencing pesticides, a new provision clarifies that techniques involving the application of RNA to an organism to temporarily induce RNAi do not constitute gene technology, provided that the RNA cannot be translated into a polypeptide, the organism's genomic sequence cannot be altered as a result, and an infectious agent cannot be produced.

When these conditions are satisfied, the resulting organisms are not GMOs for the purposes of the *Gene Technology Act 2000*. Therefore, RNAi techniques which involve directly applying RNAs to plants for temporarily inducing RNAi have not been subject to regulation by the OGTR since 8th October 2019.

The APVMA will continue to provide regulatory oversight of topically applied RNAi-based products in Australia. Under the *Agricultural and Veterinary Chemicals Code Act 1994*, dsRNA-based products applied topically to protect plants against insect, fungal and viral pests are defined as agricultural chemical products. Data packages in support of the registration of novel agricultural chemical products address, at a minimum, chemistry and manufacture, human health, worker health and safety, environmental fate and toxicity, efficacy and crop safety, and overseas trade. The submitted data for each of these areas should be of sufficient quality for the study to be relied upon for regulatory decision-making. For the APVMA to grant an approval or registration, the APVMA must be satisfied that the safety, trade, and efficacy criteria relevant to the particular active constituent or

product are met. Presently, specific guidelines about the types of information that can be submitted to address these criteria for topically applied RNAi-based products are not available; however, the APVMA provides pre-application assistance to prospective applicants, and this service is invaluable for new technologies such as RNA-induced gene silencing pesticides.

CONCLUSION

The potential benefits of deploying topically-applied RNAi as a crop protection measure are many, including low toxicity relative to many existing pesticides, species-specificity, and a nominal environmental impact with appropriate dsRNA design. Realizing many of these benefits is however dependent on the development of delivery mechanisms with a similarly light footprint. As with any new technology, there are identified risks that should be avoided in the first instance, and mitigated in the second. Putative unintended consequences primarily relate to impacts on human health and the environment. Given the multiple and redundant barriers to uptake of dsRNA by humans, it appears unlikely that significant deleterious impacts would become evident upon exposure. The ability for dsRNA to rapidly degrade in the environment presumably limits its impact to non-target organisms both at the point of application and post-application. Closely related species to the target species are the most likely to be affected due to their genetic similarity and probable susceptibility to environmental RNAi if they are present in the close vicinity of the application. Bioinformatics-based design of dsRNA sequences to minimise homology with endogenous transcripts in both the host plant and NTOs is an important approach to avoiding and mitigating risks. Limitations of this approach however necessitate it should be part of a suite of tools that help ameliorate any unforeseen consequences for environmental impacts. If conception and development is conducted in a precautionary and rigorous way, RNAi-based products have the ability to revolutionize pest and pathogen management in a safe and effective manner.

AUTHOR CONTRIBUTIONS

SF, PR, and NM wrote the manuscript. BH and SF designed the figures. All authors contributed to the conception, revision, editing and approval of the manuscript.

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Problem Formulation for Off-Target Effects of Externally Applied Double-Stranded RNA-Based Products for Pest Control

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Externally applied dsRNA-based biocontrol products may lead to off-target degradation of messenger RNA in target and non-target organisms. For the purposes of regulatory risk assessment of such products, producing a comprehensive catalog of any off-target effects using profiling methods is unnecessary and would be ineffective in supporting decision-making. Instead, problem formulation should derive criteria that indicate acceptable risk and devise a plan to test the hypothesis that the product meets those criteria. The key to effective risk assessment of dsRNA-based biocontrols is determining whether their properties indicate acceptable or unacceptable risk, not whether they arise from on- or off-target effects of dsRNA.

Keywords: hypothesis testing, problem formulation, acceptable risk, decision-making, targeted risk assessment

INTRODUCTION

Double-stranded (ds) RNA has roles in virus defense and immune responses in animals (Reich and Bass, 2019) and plants (Niehl et al., 2016). Among other effects, it triggers sequence-specific degradation of messenger RNA (mRNA) via RNA interference (RNAi). GM crops producing dsRNA that triggers RNAi in pests or pathogens are effective in reducing insect damage or disease (Head et al., 2017; Lindbo and Falk, 2017). dsRNA may also be effective against crop pests and pathogens when suitably formulated and applied externally to the crop. Commercial products based on this "non-transformative" technology are in development (Zotti et al., 2017). Biocontrols based on dsRNA are attractive because they are likely to pose low risk to non-target species (Bachman et al., 2016; Joga et al., 2016) and dsRNA has low persistence in the environment (Dubelman et al., 2014; Fischer et al., 2017); however, unintended silencing of transcripts ("off-target effects") raises concerns (Kulkarni et al., 2006).

Data requirements for regulatory decision-making for biocontrol products based on externally applied dsRNA are not clear (Darsan Singh et al., 2019). Nevertheless, externally applied dsRNA products will almost certainly require assessment of the acceptability of the risks that their use poses to human and animal health and the environment. These risks may arise from exposure to the dsRNA or any formulant that helps the effectiveness of the product (Christiaens et al., 2018). Problem formulation may help in the design and conduct of these assessments.

PROBLEM FORMULATION

Problem formulation organizes existing knowledge and identifies relevant new knowledge to support decision-making. Its origins are in ecological risk assessment, but it may be used in any situation where science informs decisions (Sauve-Ciencewicki et al., 2019).

Problem formulation translates policy objectives into operational decision-making criteria and devises tests of the hypothesis that the proposed activity meets those criteria. In regulatory risk assessment, policy objectives are those of the laws that the regulations are intended to implement, and hence are ultimately those of the government of the country making the decision. Policy may be thought of more broadly as the objectives of any decision-maker; thus, our remarks also refer to non-regulatory decision-making, where objectives may be those of non-governmental organizations, such as private companies or public-sector research bodies making product-development decisions. To avoid appearing to advocate particular policies, we do not define specific harmful effects. Instead, we encourage risk assessors to consult policy- and decision-makers to agree definitions of harm before beginning the risk assessment.

Problem formulation also organizes existing data to test hypotheses so that new data are acquired only if the existing data are insufficient for decision-making (Raybould, 2006). Problem formulation is conceptually straightforward, although its implementation is often difficult because the objectives of the decision are unclear or there is uncertainty about how to determine whether taking a course of action is likely to achieve stated objectives.

RISK ASSESSMENT AS HYPOTHESIS TESTING

Regulatory risk assessments for externally applied dsRNA-based biocontrol products (the dsRNA active substance and any formulants) are likely to draw on experience gained in evaluating uses of biological pesticides (Mensink and Scheepmaker, 2008; Arora et al., 2016), conventional pesticides developed through synthetic chemistry (Finizio and Villa, 2002; Boobis et al., 2008), and GM crops that produce insecticidal dsRNAs (Bachman et al., 2016; Petrick et al., 2016) or proteins (Mendelsohn et al., 2003).

Such regulatory risk assessments work well when they test a hypothesis that directly informs a decision. Risk assessment is part of risk analysis, which can be summarized as follows:

- Use aims of regulatory policy to define what risks are acceptable and unacceptable
- Derive criteria that indicate the proposed product use poses acceptable risk
- Test the hypothesis that the product use meets those criteria
- Use the results of the tests in decision-making about product-use approvals

Problem formulation comprises the setting of acceptability criteria and a plan to test that the criteria are met. Risk characterization evaluates the results of the tests. Problem formulation and risk characterization are the main elements of risk assessment.

A crucial element of problem formulation is the setting of decision-making or acceptability criteria. Toxicity: exposure ratios (TERs) used in making decisions about uses of pesticides are good examples. A TER comprises a measure of the toxicity of a pesticide to a group of organisms and an estimate of the worst-case exposure of that group of organisms when the pesticide is used properly (Damalas and Eleftherohorinos, 2011). If the TER is above a pre-set "trigger" value, risk is acceptable; if the TER is below the trigger, acceptable risk has not be shown. Being above or below the trigger leads directly to different decisions about, for example, whether to require more data to assess risk. In effect, decision-making is based on corroboration or refutation of the hypothesis that the TER is greater than the trigger.

Risk assessment is much less successful when it is data-led. Instead of testing hypotheses about whether certain acceptability criteria are met, data-led risk assessment tests the null hypothesis that the proposed activity will not result in effects that are different from a similar current activity. An example is comparing the effects of exposing organisms to a dsRNA-based biocontrol and to a suitable control substance. Any statistically significant differences are evaluated for their "biological relevance" (EFSA Scientific Committee, 2011). As discussed below, testing a null hypothesis of no difference is a method for accumulating and presenting data, not testing hypotheses that help decision-making, and is an inefficient and ineffective way to assess risk (Raybould and Macdonald, 2018; Raybould et al., 2019).

INTENDED AND UNINTENDED EFFECTS

In regulatory decision-making about GM crops, risk assessment has been hypothesis-led when considering the potential side effects of the intended modification. Examples include assessing the acceptability of risks posed to biological-control organisms from the cultivation of GM crops with insect-control traits by testing hypotheses about TERs (Romeis et al., 2008), and the acceptability of risks posed to crop production from the cultivation of herbicide-tolerant crops by testing hypotheses about the abundance of herbicide-tolerant weeds (Devos et al., 2018).

Difficulties in GM crop risk assessment have arisen when considering unintended effects of genetic modification (Filipecki and Malepszy, 2006). Instead of using problem formulation to define what unintended properties of a crop would be unacceptable, or at least undesirable, regulatory risk assessments have used a data-led (or "profiling") approach that tests for statistically significant differences between a GM crop and a suitable near-isogenic non-GM comparator. Many characteristics are compared and the degree of difference in any given character that would indicate unacceptable risk is not predetermined. Comparisons include phenotypic characterization (Horak et al., 2015) and compositional analysis studies (Herman et al., 2017), and some authors have suggested that comparisons are expanded to include transcriptomic, proteomic and metabolomics profiles (Christ et al., 2018). In the remainder of the paper, we use

the term profiling mainly to refer to molecular ("omics") methods, but we intend the term to cover all studies that compare numerous characters without predetermining acceptability criteria; hence, we consider phenotypic and compositional analyses that test null hypotheses of no difference to be profiling.

A similar situation to GM crops applies to products based on external application of dsRNA. A hypothesis-led approach for assessing risks from intended effects could use current frameworks; for example, methods for assessing non-target organism toxicity and exposure to chemical pesticides can be adapted for use with dsRNA-based biocontrol. Adaptation of these methods to dsRNA-based products is considered elsewhere (Sherman et al., 2015; Bachman et al., 2016; Haller et al., 2019) and is not considered further here.

There are suggestions that hypothesis-led approaches to risk assessment should be augmented by molecular profiling of the effects of dsRNA in tissue cultures or standard laboratory test organisms (Heinemann et al., 2011, 2013; Sherman et al., 2015). Proponents of profiling suggest that it will improve human and ecological risk assessment because we do not have a "complete understanding of the biochemistry" of dsRNA-induced RNAi (Heinemann et al., 2011). Others suggest that profiling is unnecessary in many circumstances because negligible risk can be demonstrated based on lack of "functional exposure" to dsRNA because of dietary barriers (Sherman et al., 2015).

The existence of dietary barriers may be a useful hypothesis to test in a risk assessment; however, if the hypothesis that barriers exist is refuted, resorting to profiling of unintended (off-target) effects is still unnecessary. Instead, problem formulation should be used exactly as for the assessment of side-effects of the intended ("on-target") effects of the dsRNA: devise criteria for accepting that the product use poses acceptable risk and test that those criteria are met. Effects should be judged by their potential to cause harm, not by whether they result from on- or off-target effects.

COMPARING TARGETED AND UNTARGETED ASSESSMENTS

Problem formulation produces a plan to test hypotheses that a product use meets predetermined acceptability criteria. Existing data, and new data if necessary, are sought, or "targeted," to provide rigorous tests of such hypotheses. In contrast, profiling sets no predetermined acceptability criteria, and aims to describe how a product or its use differs from an existing product or use. Tests of null hypotheses of no difference are used to present the data. As no decision-making criteria are set, all differences are potentially important; hence, data acquisition is untargeted. The differences in philosophy underlying these approaches, their practical implementation and their attitudes to new scientific developments are summarized in **Table 1**.

The quality of decisions supported by these approaches differs markedly. Because targeted approaches rely on policy aims and acceptability criteria being set first, they tend to produce clear and predictable, though not necessarily uncontroversial, decisions.

TABLE 1 | A comparison of targeted and untargeted approaches to risk assessment.

Aspect of risk assessment	Untargeted	Targeted
Underlying philosophy	Empiricism	Critical rationalism
Objective	Proof of safety	A tool to support decision-making
Hypothesis tested	No difference from comparator	No unacceptable risk
Number of endpoints	As many as possible	As few as necessary
Decision-making criteria	Sought in the data	Predetermined by policy
Output	Complete understanding	Acceptability of risk
Incorporating scientific advances	Precautionary neophilia	If it ain't broke, don't fix it
Resulting decisions	Obscure, arbitrary, disputed	Clear, predictable, accepted

In untargeted approaches, acceptability criteria and policy aims emerge only after the data are obtained; hence, decisions may appear arbitrary (Raybould and Macdonald, 2018). Given the undesirable features of decision-making based on untargeted risk assessment, we should examine why it is advocated.

Philosophy Underlying Risk Assessment

Targeted risk assessment tests hypotheses about concepts such as harm, risk and unacceptability. In regulatory risk assessments, these terms are defined by policy aims and a key part of problem formulation is understanding these aims and translating them into operational acceptability criteria. Untargeted risk assessment avoids operational definitions of harm, risk and acceptability, and instead tests for differences between, say, organisms exposed to dsRNA and those exposed to a control treatment. In using a neutral term like difference, untargeted assessment appears to follow the philosophy of empiricism: the idea that objective knowledge expands by generalizing from observations made without preconceptions (Hahn, 1965).

Targeted risk assessment is more akin to critical rationalism, which postulates that knowledge arises from trial-and-error testing of solutions to problems (Miller, 1994). In critical rationalism, preconceptions (hypotheses) are seen as unavoidable. The targeted approach makes a virtue of operationalizing explicitly value-laden terms, such as harm, risk and unacceptability, to formulate hypotheses directly related to decision-making. Objectivity arises from rigorously testing these hypotheses and disinterestedly evaluating the results. Untargeted approaches imply that risk can and should be characterized objectively without recourse to values, leading directly to "science-based" decisions (Davison, 2010).

A second important philosophical difference is that empiricism sees objective knowledge as a set of truths confirmed by sufficient observations, whereas critical rationalism regards objective knowledge as a collection of tested hypotheses that have not yet been falsified. Targeted risk assessment recognizes that all decisions will contain uncertainty; any conclusion that risk is acceptable is provisional. Untargeted risk assessment, on

the other hand, seems to imply that sufficient data will eliminate uncertainty – we can, indeed must, prove that something is safe (has zero probability of causing harm) before we allow its use. These differences lead to risk assessments that vary greatly in their ability to support decision-making.

Conduct and Use of Risk Assessments

The combination of not defining acceptable risk – because that would be an unwarranted preconception or improper bias – and seeking proof of safety leads untargeted risk assessment to pursue comprehensive descriptions (or "complete understanding"; Heinemann et al., 2013) of products and their potential effects when used. Null hypotheses of no difference between the effects of the proposed product use and a control treatment can be tested. However, such hypotheses do not express an expectation that a new product is no different from existing products; it is a device for presenting data (Stephens et al., 2007). Untargeted risk assessment therefore collects as many data (measures as many endpoints) as time, money and methods allow to make the most complete possible description of the product and its potential effects.

In seeking the best solution to the problem of how to make a good decision, the targeted approach recognizes that risk assessment should test hypotheses that pre-set acceptability criteria are met. It first organizes existing data to test the hypotheses, and only if these data are insufficient for decision-making are new data required. Consequently, targeted risk assessment collects as few data (measures as few endpoints) as necessary for decision-making.

Collecting as many data as possible rather than as few as necessary has bad effects on decision-making. The immediate decision, perhaps whether to approve a proposed use of a dsRNA-based biocontrol, will depend on whether the product has properties that indicate its proposed use poses unacceptable risk. We could think of these properties as needles in a haystack of other information about the product. Problem formulation starts by using policy objectives to define the characteristics of the needles and, based on these characteristics, designs a targeted strategy most likely to find needles should they exist (i.e., rigorous tests of the hypothesis that the product meets acceptability criteria) and keeps the haystack as small as possible. Decisions can be made effectively, because decision-making criteria have already been established (there is little ambiguity about what needles look like), and efficiently, because all data have clear relevance (they help to find needles should they exist).

Untargeted risk assessment deliberately avoids describing needles, and instead builds the biggest possible haystack. In searching through the haystack (testing a null hypothesis of no difference), it will find potential needles (statistically significant differences), but has no means of determining whether they really are needles (indicators of unacceptable risk) or merely peculiar pieces of straw (differences of insufficient consequence to be unacceptable). There is no method for making this distinction until someone defines unacceptability.

Building haystacks and leaving the characteristics of needles undefined increases the likelihood of making a bad decision. Properties of a product that would lead to unacceptable effects if it were used as proposed may be missed in the mass of data produced by the untargeted risk assessment. Hence, in refusing to use problem formulation to define indicators of unacceptable risk, untargeted risk assessment increases the probability of approving a product use that has effects that turn out to be unacceptable. Conversely, beneficial product uses may be refused because differences in a profile are deemed unacceptable based on potentially spurious statistical significance rather than their potential to cause harm.

The consequences of untargeted risk assessment go further than the immediate decision about the product. Time, money and expertise spent reviewing data of unknown relevance about product X cannot be spent evaluating product Y, which may mean that a harmful product receives inadequate scrutiny or that use of a beneficial product is delayed, depending on how decision-makers respond to limited resources. More widely still, the increased costs and uncertainty of decision-making associated with untargeted regulatory risk assessments is a disincentive to produce innovative products that require premarket approvals (Smyth et al., 2014). Hence, untargeted assessments increase risk directly through increasing the likelihood of poor decisions about current products and indirectly by discouraging the development of new, improved products.

Scientific Advances and Improving Risk Assessment

Regular demands are made for risk assessments to be improved by incorporating new profiling methods (Heinemann et al., 2011; Pott et al., 2018). However, these demands rarely, if ever, give examples of the failure of a specific risk assessment, or what risks are being underestimated. Proponents of the value of new methods of profiling to risk assessment fail to convince because they cannot show how the profiles are a better test of the hypothesis that a product has no properties that indicate unacceptable risk. As profiling is generally associated with a refusal to define unacceptability, this failure is unsurprising. A bigger haystack of data makes no improvement to risk assessment just because the data are acquired using the latest technology.

If the aim of untargeted risk assessment is a complete description ("understanding") of a product, then new profiling technology must be seen as an improvement because our description would be incomplete without its use. Hence, untargeted risk assessment will be driven by a "precautionary neophilia"; decisions cannot be made without data collected using the latest methods.

Targeted risk assessment has a more skeptical view of new measurement methods. If they provide better tests of the hypothesis that a product has no properties that indicate unacceptable risk, then, all other things being equal, they improve the risk assessment. "Better" may mean that the hypothesis that the product use meets existing acceptability criteria can be tested more rigorously, or that we could test that the product use meets new acceptability criteria should policy aims change. Often, all other things will not be equal; for example, new methods may cost more, take longer or need more expertise to use and interpret

than do existing methods. In these circumstances, use of the new method only makes sense if the value of the improvement in the decision outweighs the additional costs.

Setting policy aims must make compromises among different opinions and objectives, and evaluating options to achieve policy aims will be based on imperfect knowledge; hence, no method of decision-making can be perfect. Some people will disagree with the aims of a decision, and the decision may not achieve its aims or may have unwanted consequences, or both. If a decision clearly fails, we should try to correct it and the methods used to reach it should be evaluated and changed if necessary and feasible. However, untargeted risk assessment's focus on data means that improvements to decision-making would only be sought in technical developments. Use of problem formulation in targeted risk assessment means that improvements to decision-making would also be sought in increased clarity of policy aims and selection of better acceptability criteria. Often it is convenient for politicians to portray risk assessment as completely technical to delay decisions while new data are acquired (Mastroeni et al., 2019). Use of problem formulation and targeted assessment should reduce such sleight of hand.

Acceptance of Decisions

Increasing the amount of data to support decision-making often increases controversy because supporters of different views are more able to select data that support their argument (Sarewitz, 2004). The heart of this problem is the inability or unwillingness to argue about fundamental values on which opinions are based. Instead, data are used to try to prove that certain opinions are factually incorrect.

The use of untargeted risk assessment poses similar problems. When statistically significant differences are found, they have to be evaluated. Initially, evaluation may comprise a scientific study of the effects of the differences. However, at some point, a decision must be made about whether or not the effects indicate unacceptable risk. In untargeted risk assessment, selection of endpoints based on their ability to indicate unacceptability of risk is deliberately avoided. Consequently, there is no debate about fundamental values that underlie definitions of harm, risk and unacceptability, and so there is no clarity about why certain statistically significant differences are acceptable or unacceptable, and decision-making appears arbitrary. In effect, policy objectives are set by levels of statistical significance, which may be spurious given the numerous endpoints that profiling produces.

Targeted risk assessment should be less prone to such problems. Problem formulation takes the aims of policy and translates them into acceptability criteria. Good policymaking seeks to understand and reconcile opinions based on different values, or at least be clear why it favors one opinion over others. Having such clarity at the beginning means that the results of the risk assessment lead to understandable and predictable decisions. Not everyone will agree with the aims of the decision, but at least the aims will be based on what has been deemed best for society and not on an arbitrary determination of whether a statistically significant difference is "biologically relevant." Bringing policy disputes to the fore in targeted risk assessment, rather than ignoring them in a futile search for complete understanding, may help to reduce controversies about the use of new technology in agriculture (Carolan, 2008).

EFFECTIVE USE OF PROFILING FOR RISK ASSESSMENT OF DSRNA-BASED PRODUCTS

Conceivably, profiling could be useful in risk assessment if an unacceptable profile could be defined by problem formulation. Research may show, for example, that a particular profile in a tissue culture exposed to candidate dsRNAs indicates with high accuracy that the dsRNA would have unacceptable effects in a standard toxicity test using a non-target organism. Whether to replace the toxicity test with a profile would depend on several factors including the reliability and cost of each type of study, and the ethics of continuing to test animals when other options are available (Kroeger, 2006). If a profile were chosen as the decision-making endpoint, its usefulness would come from a prior definition of acceptability, not from an untargeted search for differences. We emphasize that searching for profiles that constitute useful decision-making criteria is a task for basic and applied research and should not be part of product risk assessment.

CONCLUSION

Externally applied dsRNA-based biocontrol products may lead to off-target degradation of mRNA in target and non-target organisms. For the purposes of risk assessment, a comprehensive description of any off-target effects using profiling methods is unnecessary. Instead, problem formulation should derive criteria that indicate acceptable risk and devise a plan to test the hypothesis that the product meets these criteria. The key to effective risk assessment is determining whether product's properties are acceptable or unacceptable, not whether they arise from on- or off-target effects of dsRNA.

AUTHOR CONTRIBUTIONS

AR and AB devised and wrote the manuscript.

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REVIEW
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Double-Stranded RNA Technology to Control Insect Pests: Current Status and Challenges

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Exploiting the RNA interference (RNAi) gene mechanism to silence essential genes in pest insects, leading to toxic effects, has surfaced as a promising new control strategy in the past decade. While the first commercial RNAi-based products are currently coming to market, the application against a wide range of insect species is still hindered by a number of challenges. In this review, we discuss the current status of these RNAi-based products and the different delivery strategies by which insects can be targeted by the RNAi-triggering double-stranded RNA (dsRNA) molecules. Furthermore, this review also addresses a number of physiological and cellular barriers, which can lead to decreased RNAi efficacy in insects. Finally, novel non-transgenic delivery technologies, such as polymer or liposomic nanoparticles, peptide-based delivery vehicles and viral-like particles, are also discussed, as these could overcome these barriers and lead to effective RNAi-based pest control.

Keywords: RNA interference, RNAi, pest management, insect pests, dsRNA, host-induced gene silencing (HIGS), spray-induced gene silencing (SIGS), virus-induced gene silencing (VIGS)

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INTRODUCTION

Insects are our most serious competitors for food and fiber and are vectors of some of our most serious diseases. Chemical pesticides are routinely used to protect crops and to reduce the spread of insect-borne diseases. Due to their frequent use, there are increasing incidences of insecticide resistance to many of the most commonly used insecticides (Sparks and Nauen, 2015). In addition, there is increasing public concern over the risk that many of these chemicals pose to the environment and to human and livestock health (Damalas and Eleftherohorinos, 2011; Nicolopoulou-Stamati et al., 2016). Together, these issues provide compelling reasons to find safer, more pest-specific alternatives to control pest insects. One technology that offers the promise of a reduced risk approach to insect pest control is RNA interference (RNAi). RNAi is a sequence-specific method of suppressing a targeted gene's expression, and because each species is defined by the uniqueness of its genes' sequences, RNAi can potentially be designed in a species-specific manner. By targeting genes essential for pest insect's growth, development, or reproduction, RNAi could be used selectively to kill pest insects without adversely affecting non-target species (Whyard et al., 2009).

RNAi is a naturally occurring cellular defense system mediated by double-stranded RNA (dsRNA). In most eukaryotes, long dsRNA found within a cell is seen as either a source of viral infection or as evidence of transposon activity, both of which the cell will seek to suppress

(Obbard et al., 2008). The first component of the RNAi machinery to respond to the dsRNA is the RNase III endonuclease Dicer-2 (Dcr-2), which cleaves the dsRNA into short (typically 19-21 nt long) interfering RNAs (siRNAs). Dicer-2, with the help of dsRNA-binding proteins such as R2D2, facilitates the transfer of the siRNA to the RNA−induced silencing complex (RISC). Within RISC, the siRNA is unwound, and one strand, the passenger strand, is eliminated. Using the retained guide strand, the activated RISC complex scans cellular mRNAs, and an Argonaute protein (Ago2) within RISC cleaves transcripts with complementarity to the siRNA, thus silencing gene expression (Okamura et al., 2004).

Due largely to this sequence specificity, growing numbers of research groups and biotechnology industries are exploring the efficacy of using dsRNA as a new source of environmentally friendly, potentially species-specific insecticides. Some insects, particularly of the order Coleoptera (beetles), have proven highly susceptible to dsRNA (Baum and Roberts, 2014), such that only small quantities of ingested dsRNA can induce RNAi, causing both transcript knockdown, and where essential genes were targeted, insect mortality. A particularly intriguing aspect of RNAi is that in these highly susceptible insects, the dsRNA is not only capable of entering gut cells, but can spread to other tissues to induce systemic RNAi (Joga et al., 2016). The systemic nature of RNAi is particularly useful in the development of a broader range of potential insecticidal dsRNAs that can target essential genes in many other tissues of the pest insects (Huvenne and Smagghe, 2010).

Not all insects, however, respond equally well to ingested dsRNA. Insects of the order Lepidoptera (moths and butterflies), Diptera (flies and mosquitoes), and Hemiptera (aphids, hoppers, stinkbugs), respond to dsRNA with greater variability than that seen in beetles (Cooper et al., 2019). If RNAi is to be developed for insecticidal applications in a broader range of insects, it is important that we understand some of the barriers to efficient RNAi, and consider how we might deliver dsRNA to different insects to maximize the potential of RNAi for insect control more fully. In this review, we will explore the potential for dsRNA-based insecticides by considering the methods that have been used to date to deliver dsRNA, what barriers can limit RNAi efficiency in some insects, and how alternative delivery methods may help overcome some of the limitations in certain insects.

APPLICATION OF RNAI IN THE FIELD

Application of RNAi in agriculture, more specifically in pest or pathogen control, can be achieved in different ways, namely by host-induced gene silencing (HIGS), spray-induced gene silencing (SIGS) or virus-induced gene silencing (VIGS).

HIGS entails the creation of transgenic crops that express the dsRNA specific for the pest or pathogen. The first commercial RNAi product targeting an insect pest is a transgenic corn crop, developed by Monsanto (currently Bayer CropScience), which expresses a hairpin dsRNA targeting the *snf7* gene in the Western corn rootworm, *Diabrotica virgifera virgifera* (Bolognesi et al., 2012; Bachman et al., 2013). This new RNAi construct is also stacked with two *Bacillus thuringiensis* Cry proteins (Cry3Bb1 and Cry34/35Ab), in an effort to delay the evolution of resistance (Head et al., 2017). This product will be marketed under the trade name of SmartStax Pro, was approved in 2017 by the United States Environmental Protection Agency (EPA, 2017), and is expected to be released for commercial use by the end of the decade. SmartStax Pro is considered a milestone in the use of RNAi technology in agriculture (Head et al., 2017).

Other genes have also demonstrated plant protection against *D. v. virgifera*, including the vacuolar proton pump, *V-ATPase A* (Baum et al., 2007), the septate junction proteins *snakeskin* (*ssj1*) and *mesh* (*ssj2*) (Hu et al., 2016), *Troponin I* (Fishilevich et al., 2019), SNARE binding protein *Ras opposite/Sec1*, RNA polymerase II subunit *RpII140*, FACT complex protein dre4/spt16 (Knorr et al., 2018), and *Sec23* subunit of the coat protein complex II (COPII) (Vélez et al., 2019). HIGS in other insects has been explored with a high degree of variability in the response (Yu et al., 2016; Zhang et al., 2017).

VIGS is a rather novel delivery method that is based on viruses engineered to produce the desired dsRNA in the pest itself (Kolliopoulou et al., 2017). For example, an insect virus could be modified to contain an insect-specific sequence in its genome, homologous to an insect's essential gene. Infection and replication of the virus would then lead to the production of dsRNA molecules directly in the insect cells. A major advantage of this delivery method is that a very high efficiency can be achieved, even in otherwise recalcitrant cells. Relying on the virus's own infection processes, physiological and cellular barriers for the uptake of dsRNA from the environment are thus bypassed. Furthermore, viruses can be very host-specific, thereby providing another layer of species-specificity to this technology. A proof-of-concept of VIGS directed against insects was recently provided by Taning et al. (2018), who successfully modified Flock house virus (FHV) to express *Drosophila melanogaster*-specific dsRNA.

A VIGS-like technology has also been proposed using various microbes, such as bacteria, yeast, or fungi that are engineered to serve as vectors for gene-silencing induction through the continuous production of si/dsRNA into the host (Whitten et al., 2016). A review of the use of bacteria and viruses for dsRNA delivery is provided in Joga et al. (2016) and Zotti et al. (2018). The potential, successes and concerns on micro-organisms or derived products as delivery methods for insect and disease management, are discussed in more detail in a later section.

Finally, many efforts have also focused on the use of non-transgenic, spray-based pesticidal dsRNAs (SIGS) to control pests and pathogens. SIGS can also be used for root absorption and trunk injections, where insects can acquire dsRNA through sucking and chewing, a review of this delivery method is provided in Joga et al. (2016) and Zotti et al. (2018). Given the low persistence of dsRNA molecules in the environment, SIGS will most likely need special formulations to increase the stability, and if possible, also increase the RNAi efficacy in the insect. Furthermore, the exposure of target pests through SIGS is likely to be lower compared to transgenic plants, since plants offer the possibility of continuous high expression of the insecticidal dsRNA. Therefore, spray-based applications might only become

a reality for those insects that are more sensitive to dietary uptake of dsRNA.

In the following sections, the variation in RNAi responses between insects will be discussed, focusing on physiological and cellular barriers that affect RNAi efficacy. In the last section, we will focus on formulations and delivery methods that could improve non-transgenic spray-based RNAi approaches and eventually perhaps lead to effective and sustainable RNAi-based control strategies against pests and pathogens.

VARIATION IN RNAi RESPONSE BETWEEN INSECTS

The ability of insects to acquire dsRNA through feeding (i.e., environmental RNAi) will determine the potential use of RNAi technology for insect pest management. However, different insect orders respond differently to dsRNA. From the various insects studied to date, coleopterans are, in general, highly sensitive to RNAi, while Hemiptera, Orthoptera, Diptera, Hymenoptera, and Lepidoptera have different levels of variability in their responses (**Table 1**). Multiple mechanisms appear to affect the efficiency of RNAi in different insect species, including: (1) instability of dsRNA before and after it enters the insect; (2) insufficient dsRNA internalization; (3) deficient RNAi machinery; (4) impaired systemic spreading; and (5) refractory gene targets. Cooper et al. (2019) provide an extensive review of this topic. There are not only differences in the responses across orders, but also within species, life stages, tissues, and genes (Terenius et al., 2011; Wynant et al., 2012; Guo et al., 2015; Pereira et al., 2016; Singh et al., 2017; Vogel et al., 2018; Cooper et al., 2019; Grover et al., 2019).

As noted earlier, the delivery of dsRNA for insect pest management could be through expression in transformed plants, microbes or delivery as a spray-based insecticidal dsRNA. Regardless of the delivery mechanisms, the dsRNA must be stable before it is consumed by the insect to generate an effect. For spray-based insecticides, factors such as UV light and microorganisms can degrade naked dsRNA in the environment. Whereas rain can hydrate dsRNA, making it less stable (**Figure 1**). In the next section, the strategies used to overcome these issues are described. The dsRNA may not only be destabilized by environmental factors, but its availability to feeding insects could also be impaired by binding to environmental molecules that interfere with cellular uptake. For example, in honey bee larvae, RNAi efficacy was reduced as the dsRNA was bound to the main ingredient of larval diet, royal jelly. Furthermore, when *D. v. virgifera* adults were fed with an artificial diet treated with royal jelly containing a lethal concentration of *D. v. virgifera vATPase-A* dsRNA, no mortality was observed (Vélez et al., 2016).

Once the insect has consumed the dsRNA, the dsRNA must avoid the degradation by nucleases from salivary glands, midgut, and hemolymph of the insect (**Figure 1**). Studies with hemipterans, including the tarnished plant bug, *Lygus lineolaris*, and the peach aphid, *Acyrthosiphon pisum,* have shown that dsRNA is degraded by saliva (Allen and Walker, 2012; Christiaens et al., 2014). Similarly, research performed with the tobacco hornworm, *Manduca sexta*, and the German cockroach, *Blatella germanica*, demonstrated that dsRNA degraded in the hemolymph after 1 and 24 h, respectively (Garbutt et al., 2013). Studies performed with the silkworm, *Bombyx mori*, the desert locust, *Schistocerca gregaria*, and the Colorado potato beetle, *Leptinotarsa decemlineata*, also demonstrated that midgut juices degrade dsRNA (Liu et al., 2013; Spit et al., 2017). In *B. mori*, dsRNA degraded within only ten minutes of exposure to midgut nucleases (Liu et al., 2013). The efficiency of nucleases within insect guts can vary from one species to the next. For example, 10-minute *in vitro* incubations of dsRNA with serial dilutions of gut juices showed that dsRNA disappeared much faster in *S. gregaria* compared to *L. decemlineata.* Similarly, a comparative study between two weevil species belonging to the genus Cylas, indicated that dsRNA degradation in the gut could be a source of variability, even between two very closely related species (Christiaens et al., 2016; Prentice et al., 2017). Furthermore, a study demonstrated that *L. decemlineata* with knockdown of nucleases incur less damage on potato plants expressing dsRNA (Spit et al., 2017), similar findings were observed in the sweetpotato weevil *Cylas puncticollis* (Prentice et al., 2019). These studies suggest that combining the knockdown of nucleases and a lethal gene can improve the use of RNAi as a strategy for plant protection. The variability in the stability of dsRNA in different parts of the insect body (e.g., midgut vs. hemolymph), could also be explained by differences in physiological pH that could affect dsRNA stability and nucleases' enzymatic activity. ssRNA is most stable at pH 4.0–5.0, while it is susceptible to hydrolysis at pH $>$ 6.0 and $<$2.0, and to depurination at $<$3.0 (**Figure 1**; Cooper et al., 2019). However, no experimental evidence is available so far to determine the effect of physiological pH on dsRNA stability and the activity of nucleases.

After the dsRNA has overcome the initial barriers of dsRNA degradation in the environment, external and internal, the next barrier is the internalization of the dsRNA in the cell (**Figure 2**). Two mechanisms of cellular uptake of dsRNA have been identified in insects: Sid-like transmembrane channels, and clathrin-dependent endocytosis (**Table 1**). The role of Sid-like transmembrane channels dsRNA uptake was first described in the nematode *Caenorhabditis elegans* (Winston et al., 2007; Whangbo and Hunter, 2008). In insects, Sid-like genes have been identified in Coleoptera, Hemiptera, and Lepidoptera, but the role in cellular uptake has not been directly evidenced to date (Tomoyasu et al., 2008; Xu et al., 2013; Cappelle et al., 2016; Pinheiro et al., 2018). Whereas, clathrin-dependent endocytosis seems to play the primary role in the uptake of dsRNA in multiple insects (Saleh et al., 2006; Xiao et al., 2015; Cappelle et al., 2016; Pinheiro et al., 2018). Other mechanisms involved in dsRNA/siRNA uptake in mammals such as caveolar endocytosis and micropinocytosis remain unexplored in insects. Vélez and Fishilevich (2018) provide a review of the evidence that supports the key role of endocytosis in the uptake of dsRNA and discusses the role of other components of the cellular membrane transport in the efficiency of RNAi.

Uptake of dsRNA is also affected by the dsRNA length and structure, and the vehicle used to deliver the dsRNA. For example, in *D. v. virgifera*, uptake of naked dsRNA is

TABLE 1 | Mechanisms of dsRNA cellular uptake identified in different insect species.

Order	Species	Environmental RNAi	Sid-1	Endocytosis	References
Diptera	*Drosophila melanogaster*	+	No	Yes	Saleh et al., 2006
	Bactrocera dorsalis	+	No	Yes	Li X. X. et al., 2015
Coleoptera	*Tribolium castaneum*	+	No	Yes	Tomoyasu et al., 2008; Xiao et al., 2015
	Diabrotica virgifera virgifera	++	Yes	Yes	Miyata et al., 2014; Pinheiro et al., 2018
	Leptinotarsa decemlineata	++	Yes	Yes	Cappelle et al., 2016
Lepidoptera	*Spodoptera frugiperda*	+ but no endosomal release	Not determined	Yes	Yoon et al., 2017
	Bombyx mori	–	No	Not determined	Tomoyasu et al., 2008
Orthoptera	*Schistocerca gregaria*	–	No	Yes	Wynant et al., 2014
	Locusta migratoria	–	No	Not determined	Luo et al., 2012
Hymenoptera	*Apis mellifera*	+	Yes	Not determined	Aronstein et al., 2006
Hemiptera	*Nilaparvata lugens*	–	Yes	Not determined	Xu et al., 2013

Adapted from Cappelle et al. (2016). RNAi: ++, present and robust; +, present but not robust; –, not present.

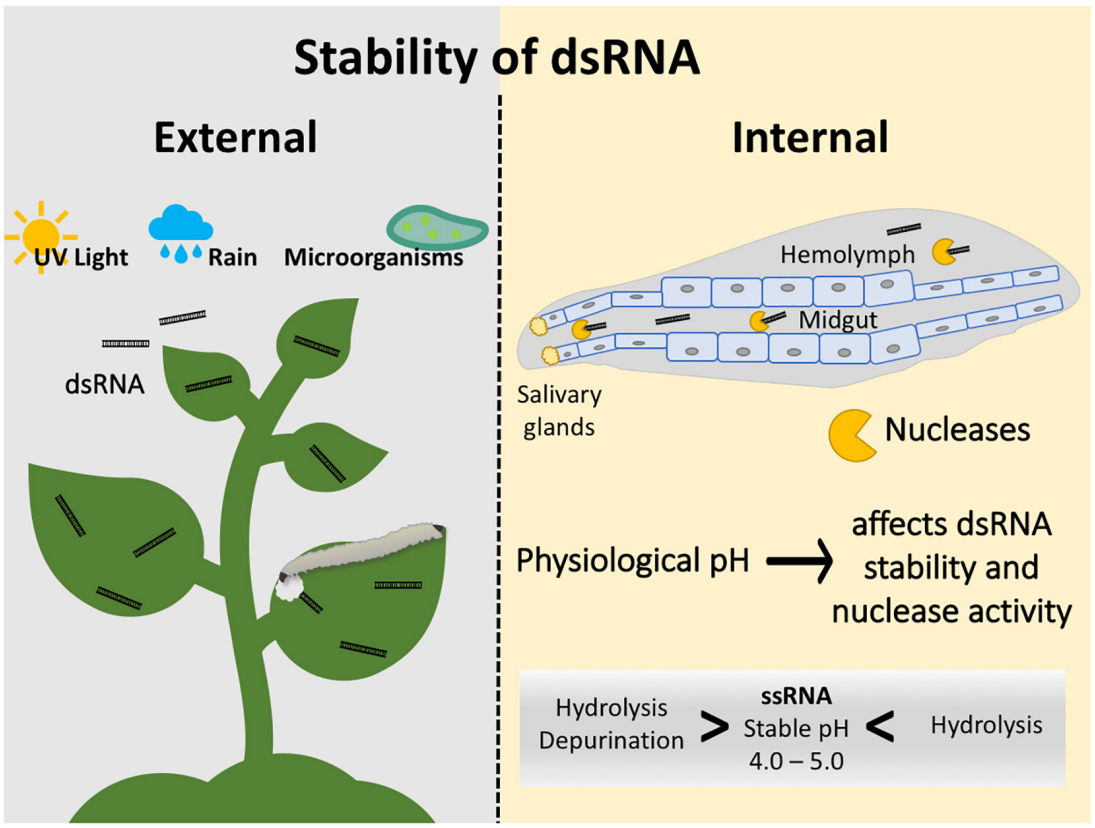

FIGURE 1 | Factors affecting the stability of dsRNA in the environment and inside the insect. External factors include degradation by UV light and microorganisms and runoff of sprayable dsRNAs by rain. Internal factors include nucleases present in salivary glands, midgut, and hemolymph. Physiological pH affects dsRNA stability and nuclease activity; ssRNA is stable at a pH of 4.0–5.0.

limited to long dsRNA, no shorter than 60 bp (Bolognesi et al., 2012; Li H. et al., 2015). Several chemical modifications of dsRNA and vehicles of delivery are discussed in the next section. Once the dsRNA enters the cell through endocytosis, the dsRNA needs to be released from the endosome to get in contact with the RNAi machinery (i.e., *dcr-2* and RISC) and generate knockdown of the targeted gene (Saleh et al., 2006; Xiao et al., 2015). Endosomal release occurs after the endosome is acidified. Research performed with the fall armyworm, *Spodoptera frugiperda*, demonstrated that the lack of endosomal release of the dsRNA leads to low sensitivity to RNAi in Lepidoptera (**Figure 2**; Shukla et al., 2016; Yoon et al., 2017). Another example of the potential limitation of uptake in RNAi efficiency is the identification of *D. v. virgifera* resistant to *snf7* dsRNA. Resistance to *snf7* dsRNA showed cross-resistance to other dsRNAs, and microscopy experiments

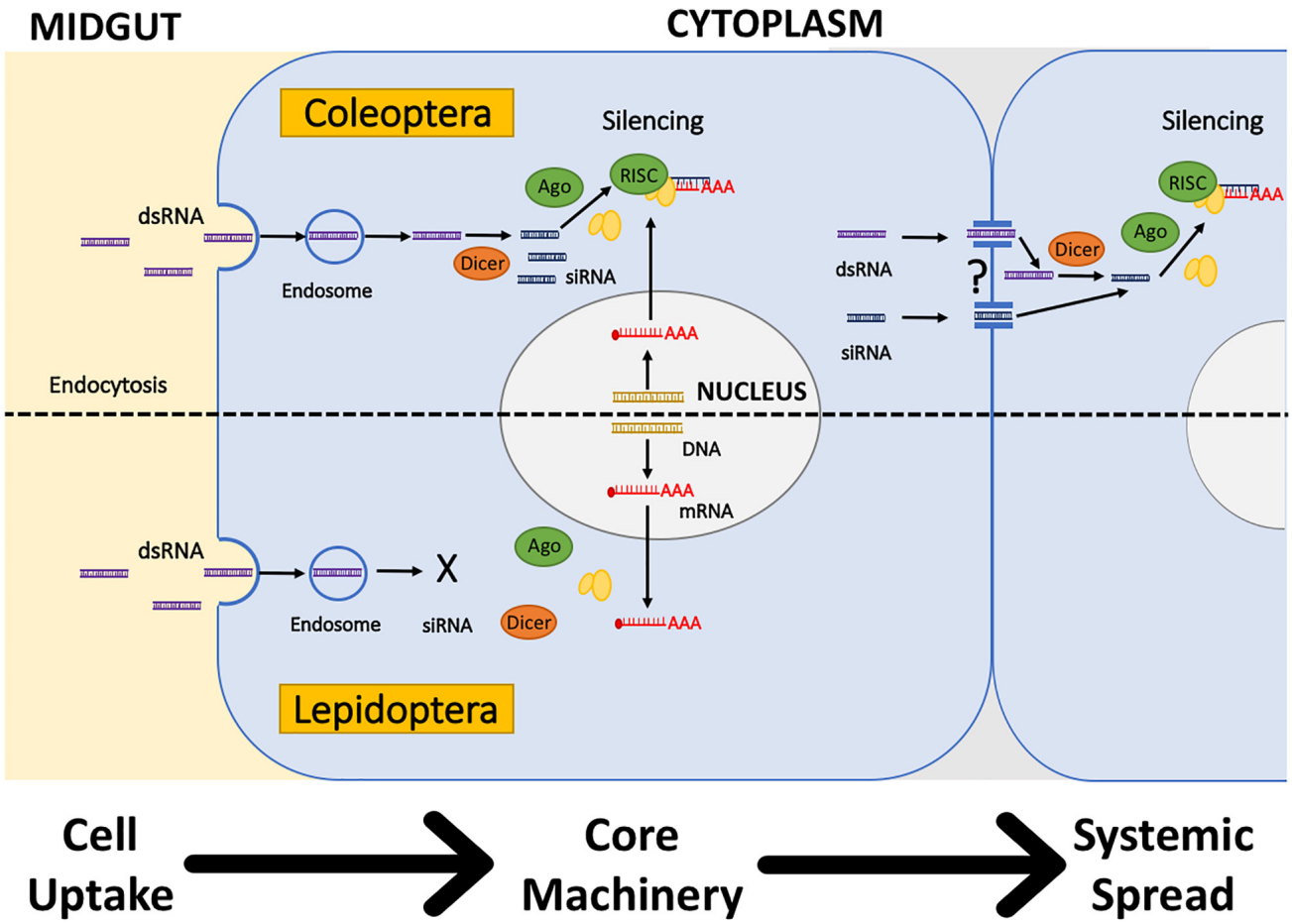

FIGURE 2 | Hypothetical differences in the cellular internalization, processing, and systemic spread of dsRNA in Coleoptera **(Top)** and Lepidoptera **(Bottom)**. *Cell Uptake and Processing*: Clathrin-dependent endocytosis is hypothesized to be the primary dsRNA uptake mechanism in insects. In Coleoptera, dsRNA is released from the endosome and processed by the core RNAi machinery to silence mRNA. In Lepidoptera, dsRNA is not released from the endosome, avoiding dsRNA contact with the core RNAi machinery. *Systemic Spread*: In Coleoptera, experimental evidence suggests that systemic spread occurs, but is not clear if it is in the form of dsRNA or siRNA. In Lepidoptera, no current evidence exists regarding the systemic spread. Adapted from Shukla et al. (2016).

determined that resistance was linked to the uptake of dsRNA (Khajuria et al., 2018).

After the release of the dsRNA from the endosomes, the dsRNA is processed by the RNAi (core) machinery to generate sequence-specific gene knockdown (Okamura et al., 2004). In eukaryotes, three RNAi pathways have been described: (1) siRNA consisting of an exogenous and endogenous pathway for viral and transposon defense, respectively; (2) microRNA (miRNA) a pathway that regulates gene expression at the transcription level, and (3) piwi-interacting RNA (piRNA) which functions in the epigenetic control of genomic elements (Kingsolver et al., 2013). While the RNAi mechanism is conserved across eukaryotes, differences in the proteins involved in the core machinery of the three different pathways vary between clades. Plants have four Dicer-like proteins, while insects have two, and annelids, nematodes, mollusks, and higher animals only have one (Mukherjee et al., 2013). Ago-like proteins are even more diverse, with insects having four (Ago1, Ago2, Ago3, Piwi, and Aubergine), humans have eight, and *Arabidopsis thaliana* plants have ten (Hock and Meister, 2008). In insects, the different pathways involve different proteins, including different Dicer, Ago, and other ancillary proteins (Cooper et al., 2019). When thinking about RNAi efficiency, it is useful to think about the duplication of core RNAi pathway genes (Tomoyasu et al., 2008; Guo et al., 2015). Yoon et al. (2016) demonstrated that *ago1*, *ago2,* and *aubergine* were essential for RNAi in *L. decemlineata* cell line. Interestingly, *ago1* and *aubergine* are part of the miRNA and piRNA pathways, respectively. Other components of the miRNA pathway also seemed to play a partial role in the siRNA pathway. The results of this study suggest that gene duplication might explain the effectiveness of RNAi in Coleoptera. However, the involvement of miRNA and piRNA in dsRNA-mediated RNAi needs to be further investigated in Coleoptera and other insects (Yoon et al., 2016).

In addition to gene duplication, the baseline mRNA expression of core machinery genes could also explain the

differences in the RNAi efficiency between different insect orders. To test this hypothesis, Davis-Vogel et al. (2018) evaluated eight proteins from the siRNA and miRNA pathways among three agricultural pests from three different orders: *D. v. virgifera* (Coleoptera), *S. frugiperda* (Lepidoptera), and *Nezara viridula* (Hemiptera). In this study, researchers compared transcript levels of core machinery proteins Drosha, Dcr-1, Dcr-2, Pasha, Loquacious, R2D2, Ago-1, and Ago2 among the three species. Direct comparison of the proteins in the three insects revealed that *D. v. virgifera* had an increase in *loquacious* expression, an insect with a robust RNAi response (Davis-Vogel et al., 2018). In a different study, low *r2d2* gene expression was suggested as one of the reasons for a *B. mori* ovarian cell line insensitivity to RNAi (Swevers et al., 2011). These studies suggest that differential gene expression of core machinery genes might influence the RNAi response in insects, but further evidence is needed.

Maximizing the utility of RNAi in insects requires the systemic spread of the RNAi response throughout the insect body. A strong systemic response requires a sufficient number of siRNAs to reach a high number of cells in the insect body. In *C. elegans*, the RNA-dependent RNA Polymerase (RdRP) generates secondary siRNAs from the primary siRNA (Sijen et al., 2001). However, RdRP in arthropods is restricted to the tick lineage and is not found in insects (Gordon and Waterhouse, 2007). In insects, evidence of a systemic RNAi response has only been indirectly determined by observing gene knockdown in tissues distant from the place of uptake (i.e., hemolymph or gut) (Bolognesi et al., 2012; Ivashuta et al., 2015; Khajuria et al., 2015; Niu et al., 2017; Li et al., 2018). Only one study in *D. v. virgifera* has shown the spread of the RNAi response using microscopy. Researchers reported the reduction of mRNA molecules in gut and fat body, but there was no detection of secondary siRNA production, suggesting that the origin of siRNAs is restricted to the processing of the initial dose of dsRNA (Li et al., 2018). Even though systemic RNAi is observed in insects, the specific mechanisms, genes involved in the spread of the dsRNA, and the form of the signal (dsRNA or siRNA) are yet to be unraveled (Vélez and Fishilevich, 2018). Two mechanisms of transport of dsRNA between cells have been suggested in the context of viral infection: (1) *via* derived complementary viral DNAs (vDNA) used as template for *de novo* synthesis of secondary viral siRNAs (vsRNAs) released in exosomes (Tassetto et al., 2017); and (2) through nanotube-like structures observed in *D. melanogaster* cultured cells (Karlikow et al., 2016). Further research on systemic RNAi will provide insights to improve RNAi use in pest management in other insect orders.

Finally, another factor that has been described to interfere with RNAi efficiency is the presence of viruses in the targeted insect. Since the RNAi pathway is an antiviral defense mechanism, viruses can influence the core machinery availability (Christiaens and Smagghe, 2014). Furthermore, since viruses have evolved with the RNAi defenses, some of them have developed mechanisms to inhibit the RNAi proteins (Haasnoot et al., 2007). For example, in *Drosophila,* a protein from the Flock House virus binds to the dsRNA, which in turn cannot be diced by Dicer and this affects binding to the RISC complex (Chao et al., 2005). While in honey bees, injection of *GFP* dsRNA and Sindbis virus regardless of the sequence, reduced virus infection (Flenniken and Andino, 2013). Swevers et al. (2013) provide a review of the impact of virus infection on the RNAi machinery in insects.

METHODS OF DELIVERY AND FORMULATIONS

Many efforts have been made to overcome these physiological and cellular barriers in different insect species and increase RNAi efficacy in insects for non-transgenic, SIGS. These efforts range from chemical modifications of the dsRNA molecule to the use of a variety of delivery vehicles and other formulations. Recently, a study reported that the addition of EDTA as a co-formulant could increase RNAi efficacy in the Neotropical stinkbug *Euschistus heros*. First, they demonstrated *in vitro* that the addition of EDTA, which is a known inhibitor of metalloenzymes, led to increased stability of the dsRNA in *E. heros* saliva. They also observed a significant increase in RNAi-induced mortality for one of the two tested target genes (Castellanos et al., 2019).

Chemical modifications to the dsRNA (or siRNA) could also improve its stability in different environments. For example, the use of siRNAs that were modified to contain two 2'−methoxyl−nucleotides on each end of the siRNAs led to effective RNAi silencing in the diamondback moth, *Plutella xylostella* (Gong et al., 2011, 2013). Literature from the vertebrate RNAi field also suggests that chemical modifications could reduce the potential of off-target effects when using short siRNAs (Jackson et al., 2006). Several smaller industry players are now investigating the potential of chemically modified dsRNA or siRNA for pest control.

DsRNA could also be delivered by micro-organisms in order to overcome or bypass the RNAi-barriers in insects. For example, RNAi can be achieved by feeding insects with dsRNA-producing *E. coli* (Joga et al., 2016; Vatanparast and Kim, 2017). Feeding insects with dsRNA-producing bacteria could lead to a more sustained release of the dsRNA in the insect and could help avoid rapid degradation in the digestive system. RNAi efficiency and its use for pest control could even be increased further by using engineered symbionts of the target pest. Whitten et al. (2016) engineered such symbionts for two insect pests: the Western flower thrips *Frankliniella occidentalis* and the kissing bug *Rhodnius prolixus*. In both cases, a long-lasting RNAi silencing effect was observed, which was a considerable improvement compared to other feeding or injection delivery methods. Furthermore, it was observed that the symbiont was also horizontally transmitted through the population *via* feces (Whitten et al., 2016; Whitten and Dyson, 2017).

Another way to overcome some of the barriers is by using nanocarriers that could increase the stability of dsRNA in the insect body or increase cellular uptake rate of dsRNA upon ingestion. Examples of these are liposomes, polymers, and peptides. In one of the earliest studies on the potential of exogenous insecticidal dsRNA, Whyard et al. (2009)

demonstrated that feeding Lipofectamine-encapsulated dsRNA targeting essential genes could lead to an efficient gene silencing and high mortality in the fruit fly *D. melanogaster*, while naked dsRNA had no observable effect. This was later confirmed by Taning et al. (2016) in the pest fruit fly *Drosophila suzukii*, suggesting that fruit flies have an impaired cellular uptake capacity for dsRNA. Lipofectamine or other liposomic compounds have also proven their ability to improve RNAi efficacy in other insects, such as the hemipteran stinkbug *E. heros* (Castellanos et al., 2019), the cockroach *B. germanica* (Huang et al., 2018) and the tick *Rhipicephalus haemaphysaloides* (Zhang et al., 2018). Another intriguing concept is the use of so-called bacterial minicells. Although research on these vesicles for RNAi applications is scarce, certain startup companies, e.g., Agrospheres, are exploring such technology for dsRNA or siRNA delivery in the field.

Another class of promising compounds is cationic polymers. These polymers could be specifically synthesized to protect dsRNA against nucleolytic degradation at various pH conditions and could also improve cellular uptake. An early example of this was the use of the natural polymer chitosan to improve RNAi efficacy in mosquitoes (Zhang et al., 2010). Since then, many other studies have proven the potential of these carriers in other species, including *Spodoptera exigua* (Christiaens et al., 2018), *Ostrinia furnacalis* (He et al., 2013), *S. frugiperda* (Parsons et al., 2018), and *Aedes aegypti* (Lopez et al., 2019). Recently, a guanylated polymer developed at Ghent University, Belgium, was able to protect dsRNA against nucleolytic degradation in a high alkali environment and significantly improve RNAi efficacy in the lepidopteran *S. exigua* (Christiaens et al., 2018). Additionally, the polymer appeared to also improve cellular uptake of the dsRNA in lepidopteran midgut cells. While the underlying mechanism is unknown, the polymer may bypass the typical endocytic pathways known to be involved in cellular dsRNA uptake (Christiaens et al., 2018).

Recently, a non-toxic and biodegradable layered-double-hydroxide nanoparticle, called BioClay, was developed at the University of Queensland, Australia (Mitter et al., 2017). This nanoparticle could be loaded with dsRNA and leads to a sustained release, as the BioClay degrades. In their study, they opted for the delivery of a plant virus targeting dsRNA and were able to detect this dsRNA for at least 30 days after being sprayed on the plants, which was a considerable improvement compared to naked dsRNA. Functionally, it led to a successful antiviral effect in the plant for at least 20 days, which suggests that the dsRNA, either with or without the nanoparticle, is being taken up by the plant cells (Mitter et al., 2017).

Peptide- or protein-based nanoparticles could also be used as a delivery vehicle. Recently, cell-penetrating peptides (CPP) were used for the first time as a carrier for dsRNA in insects. Gillet et al. (2017) synthesized a recombinant fusion protein containing a CPP amino acid sequence fused to a dsRNA binding domain. Nanoparticles comprising a dsRNA-peptide complex significantly improved RNAi efficacy in the RNAi-insensitive cotton boll weevil *Anthonomus grandis*. This promising result should encourage the development and testing of other types of peptides or proteins for their applicability to other pest insect control systems.

Finally, one potential delivery method that has shown promise in vertebrate systems, but has not been explored in insects, is viral-like particles (VLPs). VLPs can be produced in micro-organisms and have the ability to self-assemble *in vitro*, allowing the integration of the dsRNA inside the particle (Hoffmann et al., 2016). Alternatively, the dsRNA and VLPs could also be co-expressed in bacteria, allowing immediate use in the field or purification of the dsRNA-containing particles. The advantages are similar to the use of replicating engineered viruses, in that they could allow efficient cellular uptake and protection of the dsRNA in the extracellular environments of the insect. Furthermore, they might also be able to offer a certain degree of host specificity. VLPs could be a more realistic alternative to the use of engineered viruses, since they would not have some of the biosafety or public acceptance concerns that are associated with the release of genetically modified viruses.

Further inspiration for novel dsRNA delivery methods could also be taken from the medical field, where pharmaceutical Research and Development has been searching for ways to overcome similar barriers in vertebrates. Of course, such formulations could also have an impact on food/feed safety risk assessment of these RNAi-based pest control products, so these will have to be taken into account during the risk assessment process.

CONCLUSION

RNAi continues to be considered a promising pest management strategy, largely due to its potential for environmentally friendly control. The first RNAi-based products, targeting insects that are highly sensitive to dietary uptake of dsRNA, will soon be commercially available. However, the application against a wide range of insect species is still hindered by a number of challenges. These challenges, which are largely linked to the variable RNAi sensitivity of oral RNAi in insects, are likely to be addressed by the use of different formulation strategies improving dsRNA persistence and cellular uptake in these insects. Certain proof-of-concept studies in this field have been published already and show promise, but further progress needs to be made before these RNAi products against a wide range of insect species can compete with the currently used conventional chemical pesticides. Research on the effect of nucleases and physiological pH in dsRNA stability, mechanisms of dsRNA uptake and systemic spread, interaction with viruses, and potential mechanisms of resistance will aid in improving this technology in the future.

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Risk Assessment Considerations for Genetically Modified RNAi Plants: EFSA's Activities and Perspective

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Genetically modified plants (GMPs) intended for market release can be designed to induce "gene silencing" through RNA interference (RNAi). The European Food Safety Authority (EFSA) and other international risk assessment bodies/regulatory agencies have taken several actions to determine whether the existing risk assessment approaches for GMPs are appropriate for the risk assessment of RNAi-based GMPs or require complementary or alternative approaches. To our knowledge, at the international level, no dedicated guidelines have been developed for the risk assessment and regulation of RNAi-based GMPs, confirming that existing science-based risk assessment approaches for GMPs are generally considered suitable for RNAi-based GMPs. However, some specificities have been identified for the risk assessment of RNAi-based GMPs. Here, we report on some of these specificities as identified and addressed by the EFSA GMO Panel for the molecular characterisation, food/feed safety assessment and environmental risk assessment of RNAi-based GMPs, using the DvSnf7 dsRNA-expressing maize MON87411 as a case study.

Keywords: crops, RNAi, dsRNA, DvSnf7, gene silencing, off-target, risk assessment, genetically modified organisms

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INTRODUCTION

Genetically modified plants (GMPs) and/or derived food/feed (FF) products, are subject to a risk assessment and regulatory approval before entering the market in the European Union (EU). In this process, the role of the European Food Safety Authority (EFSA) is to assess and provide scientific advice to risk managers on any possible risks that the deployment (e.g., consumption or cultivation) of GMPs may pose to humans, animals and the environment (Waigmann et al., 2012). EFSA's scientific advice on the risk assessment of GMPs is given through its scientific Panel on genetically modified organisms (GMOs) consisting of scientific experts coming from EU research institutes, universities or risk assessment bodies. For the evaluation of GMP market registration applications, EFSA's GMO Panel is supported by the GMO Unit, and three standing working groups, each of which focuses on specific risk assessment areas addressing: (a) the molecular characterisation of GMPs; (b) the FF safety assessment of GMPs and/or derived FF products; and (c) the environmental risk assessment of GMPs (see **Figure 1** for further details; Devos et al., 2014).

Plants can be engineered to induce gene silencing through RNA interference (RNAi). At present, RNAi-based GMPs have been designed to express either a double-stranded RNA (dsRNA) or an

artificial microRNA (miRNA) precursor. These molecules are cleaved by Dicer/Dicer-like proteins into a pool of small RNAs that are 20–30 nucleotides long (small interfering RNAs [siRNAs] or miRNAs) and which specifically bind the target/messenger RNA (mRNA) with perfect or nearly perfect complementarity (Burand and Hunter, 2013; Koch and Kogel, 2014; Cagliari et al., 2019). siRNAs and miRNAs bind to an Argonaute protein forming the RNAi-induced silencing complex which, based on sequence homology, targets cognate RNAs. Current RNAi-based GMPs typically express a dsRNA that is designed to either downregulate a plant endogenous mRNA (e.g., to alter nutrient composition), or a gene in pests or pathogens that infest these plants, the so-called environmental RNAi (e.g., Ivashuta et al., 2015).

Small interfering RNAs and miRNAs may also trigger silencing of genes in the plant other than the intended targets (i.e., *off-targets*) giving rise to unintended phenotypes (Casacuberta et al., 2015).

EFSA's RISK ASSESSMENT ACTIVITIES ON RNAi-BASED GMPs

The European Food Safety Authority has undertaken several activities on the risk assessment of RNAi-based GMPs to define in which areas existing risk assessment approaches for GMPs are suitable, or require complementary or alternative strategies. These include:

1. *International scientific workshop "Risk assessment considerations for RNAi-based GM plants" (4–5 June 2014, Brussels, Belgium:* At this workshop, experts from academia, risk assessment bodies, non-governmental organizations, the European Commission and the private sector identified scientific uncertainties on the level of exposure of humans, animals and the environment to dsRNA/artificial miRNA and derived small RNAs, hereafter referred to as silencing RNAs, and as well as limitations of *in silico* methods to unequivocally identify potential off-targets (European Food Safety Authority [EFSA], 2014).
2. *External scientific reports:* EFSA commissioned three external scientific reports in which relevant scientific literature was reviewed systematically to further inform the molecular characterisation, FF safety assessment and environmental risk assessment of RNAi-based GMPs, and address issues identified in the workshop. The report supporting the molecular characterisation addressed dsRNA and miRNA pathways in different species, including mammals, arthropods and plants (Pačes et al., 2017), while the FF safety report focused on the kinetics and possible effects of non-coding (nc) RNAs, including silencing RNAs, and upon ingestion by humans and animals (Dávalos et al., 2019). The report in support of the environmental risk assessment considered environmental RNAi-related aspects in arthropods, nematodes, and annelids and molluscs (Christiaens et al., 2018).
3. *Internal note on the strategy for the prediction and risk assessment of off-targets*: In 2017, EFSA's GMO Panel published an internal note[1] on the strategy to identify/predict off-targets and risk assess their potential impact in RNAi-based GMPs. It built on the available scientific knowledge and is expected to evolve with the progress of the knowledge in the field.
4. *GMO Panel opinions of RNAi-based GMPs*: EFSA's GMO Panel assessed market registration applications for the import and processing for food and feed uses of potato EH92-527-1 (including cultivation in the EU) and soybeans MON87705, 305423, MON87705 × MON89788, and 305423 × 40−3−2 (excluding cultivation) designed to downregulate plant endogenous transcripts that modulate amylose and starch content in potato tubers or fatty acid profile in soybeans (EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2006a, 2012, 2015; EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2013, 2016, respectively). More recently, the GMO Panel also assessed the maize events MON87411 and MON87427 × MON89034 × MIR162 × MON87411 that constitute cases of environmental RNAi (EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2018, 2019, respectively). Maize MON87411 expresses, among others, an insecticidal DvSnf7 dsRNA that downregulates the *Snf7* transcript in the western corn rootworm (*Diabrotica* spp.), and confers protection against this major maize pest. Some aspects of the risk assessment of maize MON87411 are further discussed below.

A complete overview of EFSA's activities is provided in **Table 1**.

RISK ASSESSMENT CONSIDERATIONS FOR RNAi-BASED PLANTS

Molecular Characterisation

RNA interference specificity is based on the sequence identity between small silencing RNAs and mRNA targets; however, other transcripts with sufficient sequence identity to the small silencing RNAs can also be targeted for destruction leading to off-target effects (European Food Safety Authority [EFSA], 2014; Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014; Ramon et al., 2014; Casacuberta et al., 2015). Thus, identifying off-targets would facilitate risk assessment. Off-targets could occur in the GMP itself, or in other organisms that are exposed to the GMP and derived products through consumption. Based on the available knowledge, EFSA's GMO Panel (see text footnote 1) considers that for plants a group of *in silico* parameters enables the prediction of off-targets, while for human and animals the available tools may not allow for sufficiently reliable predictions (Pinzón et al., 2017). Bioinformatic analyses for off-targets is based on several criteria (e.g., degree and position of base-pairing between the

[1] Available at: https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf (last accessed: 02/03/2020).

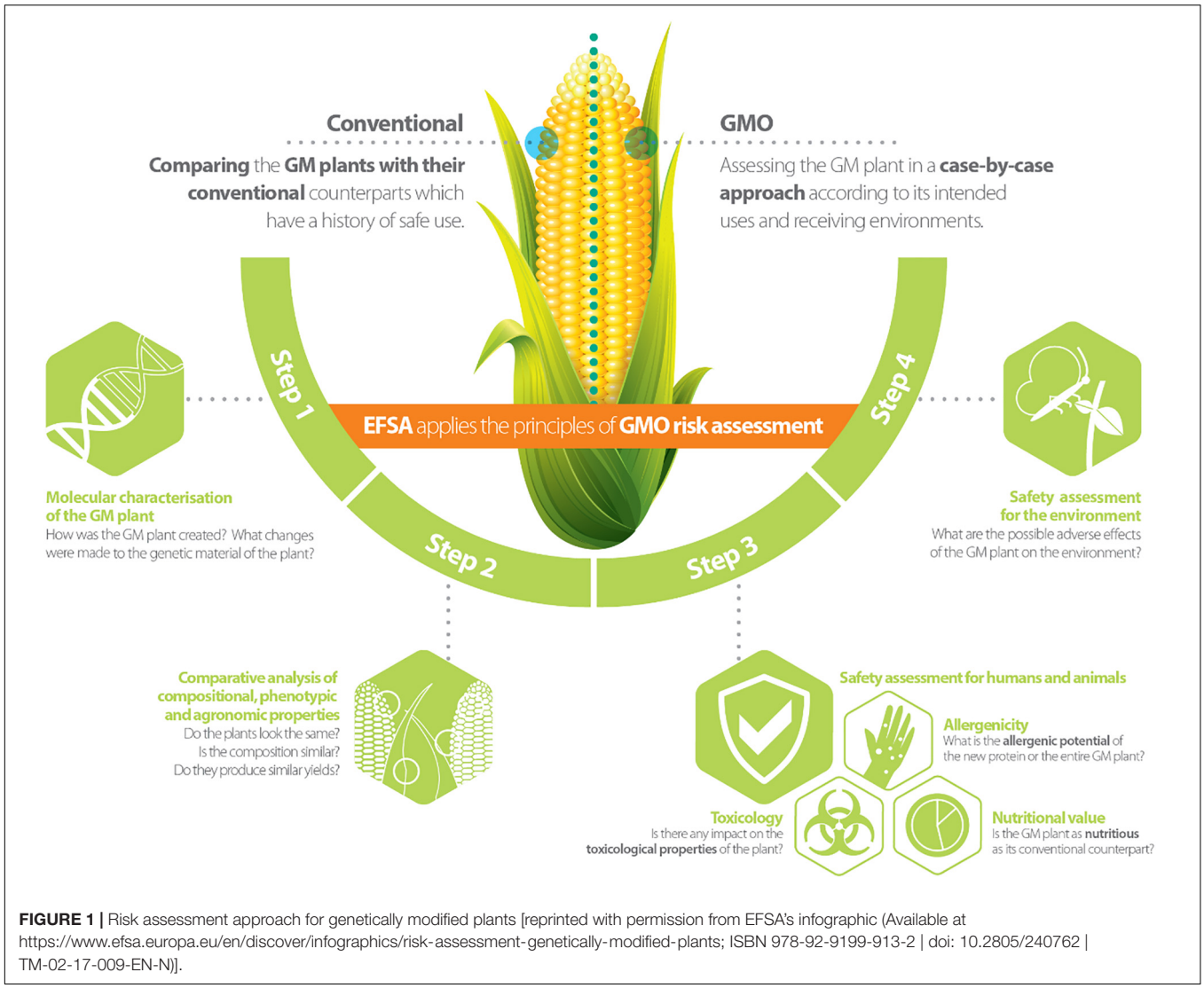

FIGURE 1 | Risk assessment approach for genetically modified plants [reprinted with permission from EFSA's infographic (Available at https://www.efsa.europa.eu/en/discover/infographics/risk-assessment-genetically-modified-plants; ISBN 978-92-9199-913-2 | doi: 10.2805/240762 | TM-02-17-009-EN-N)].

small RNA and transcript) that determine the efficiency of silencing (reviewed by Pačes et al., 2017). Therefore, *in silico* target prediction algorithms are designed based on criteria related to the biochemical and thermodynamical properties of base pairing, among other filtering parameters (Rhoades et al., 2002; Pasquinelli, 2012). In addition, other factors that can impact these interactions and lead to off-targets, is the abundance of each small RNA produced (Pačes et al., 2017). Depending on whether a dsRNA or artificial miRNA is used, a heterogeneous pool of siRNAs versus a more homogeneous pool of miRNAs will be produced, impacting the silencing of the potential off-target gene (Pačes et al., 2017).

Based on the above, the GMO Panel developed a bioinformatics-based strategy for the risk assessment of plant endogenous RNAi off-targets[1]. The parameters for identifying off-targets in plants are applicable to both siRNAs and miRNAs, and are based on a conservative approach, relying primarily on knowledge from miRNA-target specificity that accounts for complementarity mismatches between the small RNA and target gene (Liu et al., 2014). This strategy was implemented for the assessment of maize MON87411 and MON87427 × MON89034 × MIR162 × MON87411 (EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2018, 2019, respectively). The outcome of the analysis did not identify off-targets that would require further safety assessment.

Nonetheless, bioinformatic searches for potential off-targets are subject to limitations (Pačes et al., 2017). Therefore, the outcome of plant off-target analyses must take the agronomic/phenotypic and compositional field-trial data gathered as part of GMP market application into account, as they are designed to identify intended and unintended changes in GMPs. On a case-by-case basis, if a potential plant off-target is identified, additional experimental data may be needed to investigate the predicted silencing effect at transcript level (see text footnote 1).

DvSnf7 dsRNA is expressed in the plant tissues of maize MON87411 and MON87427 ×

MON89034 × MIR162 × MON87411, and induces, upon consumption by the corn rootworm, RNAi leading to pest mortality. Typically, for the molecular characterisation of GMPs, expression of new constituents (usually newly expressed proteins) is demonstrated and risk assessed with regard to FF safety. In this respect, the levels of the DvSnf7 dsRNA, have been measured in different plant tissues of maize MON87411 (Urquhart et al., 2015). However, since it is likely that plant-Dicer proteins may process some of the DvSnf7 dsRNA into siRNAs, EFSA's GMO Panel considers that "*the levels of dsRNA are not a good proxy for the levels of the active siRNAs present in plants*" (see Pačes et al., 2017; EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2018, 2019).

Food and Feed Safety Assessment

As supported by the external scientific report (Dávalos et al., 2019), ncRNAs, including silencing RNAs, are ubiquitous constituents of human and animal diet. Dietary silencing RNAs are known to be rapidly degraded soon after ingestion due to the conditions (e.g., pH) and enzymes present in the gastrointestinal tract lumen, and due to several barriers that exist at cellular (e.g., intestinal mucosa) and intracellular (e.g., lysosomal system) levels, preventing their systemic absorption. Therefore, the amount of dietary silencing RNAs absorbed after FF ingestion can be considered negligible in humans and animals (mammals, birds and fish), unless chemical modifications increasing their stability are introduced. The reported widespread presence, yet at low abundance, of exogenous RNAs in human and animal biological fluids, must therefore be viewed critically as it may be due to technical artefacts and contamination (Dávalos et al., 2019). Systemic effects of plant-derived silencing RNAs ingested orally have not been reliably established. In any case, the negligible absorption would further limit the possibility of silencing RNAs to reach a tissue or functional location in sufficient amounts and thus the possibility to exert any biological effect.

The above considerations were taken into account for the assessment of the DvSnf7 dsRNA expressed in maize MON87411 by EFSA's GMO Panel. Given that the DvSnf7 dsRNA is not chemically modified to increase stability in the plant and/or increase cellular uptake in the gastrointestinal tract and systemic absorption following oral administration, EFSA's GMO Panel concluded that the DvSnf7 dsRNA and its derived siRNAs are not able to exert any biological effects once ingested by humans and animals. Therefore, no animal studies were deemed necessary to support the FF safety assessment of maize MON87411 (EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2018). Nonetheless, Petrick et al. (2016) tested the DvSnf7 dsRNA in a 28−day oral repeated−dose toxicity study in mice and identified no adverse effects in the tested conditions.

Environmental Risk Assessment

A concern addressed for the environmental risk assessment of GMPs, including pest/pathogen-resistant dsRNA-expressing ones, for cultivation is their potential to cause harmful effects to valued non-target organisms (NTOs), especially arthropods, and the ecosystem services they contribute to (EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2010; Taning et al., 2019). For harm to occur from dsRNA-expressing plants,

TABLE 1 | Overview of the activities of the European Food Safety Authority on the risk assessment of plants genetically modified with RNA interference.

EFSA activity	Topic	References
Scientific workshop	Risk assessment considerations for RNAi-based GMPs plants	European Food Safety Authority [EFSA], 2014
External reports	Literature review of baseline information to support the risk assessment of RNAi−based GMPs	Pačes et al., 2017
	Literature review of baseline information on ncRNA to support the risk assessment of ncRNA−based GMPsfor food and feed	Dávalos et al., 2019
	Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi−based GM plants	Christiaens et al., 2018
GMO Panel Note	Internal note on the strategy for the identification/prediction and risk assessment of off-target silencing effects in plants	Annex II of the Minutes of the 118th GMO Panel plenary meeting (2017)[a]
GMO Panel scientific opinions	Assessment of potato EH92-527-1	EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2006a,b
	Assessment of soybean 305423	EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2013
	Assessment of soybean 305423 × 40-3-2	EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2016
	Assessment of soybean MON87705	EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2012
	Assessment of soybean MON87705 × MON89788	EFSA Panel on Genetically Modified Organisms [EFSA GMO], 2015
	Assessment of maize MON87411	EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2018
	Assessment of maize MON87427 × MON89034 × MIR162 × MON87411	EFSA Panel on Genetically Modified Organisms [EFSA GMO], Naegeli et al., 2019

GMP, genetically modified plant; ncRNA, non-coding RNA; RNAi, RNA interference. [a]Available at: https://www.efsa.europa.eu/sites/default/files/event/171025-m.pdf (last accessed 02/03/2020).

NTOs must be susceptible to the dsRNA expressed by the plant and ingest it in sufficient concentrations (Christiaens et al., 2018). Exposure can occur when NTOs feed on living plant material, or consume other plant parts (e.g., pollen) or plant-fed herbivores, or are exposed through plant root exudates into soil or aquatic environments (Dubelman et al., 2014; Fischer et al., 2017; Parker and Sander, 2017; Romeis et al., 2019). Once the dsRNA is ingested by the NTO, it must resist degradation in the gut, and be uptaken in sufficient quantities to activate the NTO's endogenous RNAi machinery. The latter can occur, either locally at the point of uptake (i.e., in cells lining the gut), or systemically if the NTO is able to trigger systemic RNAi (Ivashuta et al., 2015; Chan and Snow, 2017). A final condition is that the loss of the target transcript adversely affects the NTO (Bolognesi et al., 2012; Baum and Roberts, 2014). Conditions in the gastrointestinal tract of arthropods (e.g., nucleases, cellular surface receptors/membrane channels) generally do not apply to humans and food-producing animals, with the exception of crustaceans. Moreover, the efficiency of RNAi has been shown to vary greatly between different arthropod orders (Christiaens et al., 2018).

The NTO risk assessment requires consideration of the potential for off-target gene silencing (Lundgren and Duan, 2013; European Food Safety Authority [EFSA], 2014; Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014), especially for NTOs that are known to be susceptible to the dsRNA from the RNAi-based GMP and that are expected to be exposed to it. Bioinformatic analysis could identify which NTOs harbour genes that share some level of sequence homology with the target gene in the target pest/pathogen. Also, sequence complementarity between the derived siRNAs and NTO transcripts, would be indicative of potential RNAi activity in the NTO (Roberts et al., 2015; Devos et al., 2019a,b). Such data could thus be used to inform the NTO selection requiring further consideration in the risk assessment. If lack of minimum sequence homology for RNAi activity is reliably confirmed, then no further assessment may be needed (Roberts et al., 2015). However, currently, *in silico* predictions are subject to substantial limitations due to: (a) lack of sequence information for all NTOs; (b) differences between NTOs in how the RNAi machinery functions with regard to mismatches; and (c) scientific uncertainty on the exact rules governing interactions between siRNA-mRNA pairs (Ramon et al., 2014; Christiaens et al., 2018). More research on the RNAi mechanisms, design of efficient algorithms for reliable predictions and more suitable genome data for relevant NTOs will increase the usability of bioinformatic data for the assessment of off-target silencing in NTOs (Roberts et al., 2015; Christiaens et al., 2018; Devos et al., 2019a,b).

An alternative, yet complementary approach for the assessment off-targets in NTOs is to conduct laboratory bioassays with representative NTOs that are exposed to the dsRNA (Whyard et al., 2009; Bachman et al., 2013, 2016; Pan et al., 2017; Haller et al., 2019; Shang et al., 2019). Representative NTOs can include surrogate species that are selected based on their sensitivity to the dsRNA, reliability and relevance (Romeis et al., 2013). Typically, this involves phylogenetically close relatives, and species that are representative of valued taxa or functional groups that are most likely to be exposed to the dsRNA. This approach is appropriate for the assessment of RNAi effects on NTO fitness and performance, without the need for sequence information from the tested NTO. In the case of the DvSnf7 dsRNA, Bachman et al. (2013, 2016) observed no adverse effects with any of the NTOs tested at, or above, the maximum expected environmental concentration. In some cases, the timing and duration of exposure necessary to achieve the RNAi response may be uncertain, as may be the most sensitive endpoints to measure. Consequently, in some cases, and investigation of dose-dependent responses for siRNA targets may be needed (Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014; Roberts et al., 2015; Devos et al., 2019a,b).

An unresolved yet contentious point of debate is whether laboratory bioassays with plant material are useful to capture unknown complexities and variability in RNAi-based GMPs (Lundgren and Duan, 2013; Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014; Devos et al., 2016; Arpaia et al., 2017). Further evidence may be needed to investigate the usefulness and relevance of such bioassays for the assessment of unintended effects of RNAi-based GMPs for cultivation on NTOs, and what triggers their need (Devos et al., 2019a,b).

CONCLUSION

EFSA has taken several actions to determine whether the existing risk assessment approaches for GMPs are appropriate for the risk assessment of RNAi-based GMPs or require complementary or alternative approaches. Moreover, EFSA has closely followed RNAi-related activities of other international risk assessment bodies and regulatory agencies (e.g., RNAi FIFRA Scientific Advisory Panel White Paper [Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014]). To our knowledge, at the international level, no dedicated guidelines have been developed for the risk assessment and regulation of RNAi-based GMPs, confirming that existing science-based risk assessment approaches for GMPs are generally considered suitable for RNAi-based GMPs. However, the following specificities have been identified for the risk assessment of RNAi-based GMPs:

- For the molecular characterisation, EFSA's GMO Panel, along with other risk assessment bodies, considers that the identification/prediction of off-targets can be performed with a bioinformatics-based approach in plants, relying on conservative criteria, while for human and animals the available tools may not allow for sufficiently reliable predictions. Bioinformatic searches are subject to limitations and should thus be assessed in conjunction with the information derived from agronomic-phenotypic and compositional field-trials data. Furthermore, EFSA's GMO Panel does not consider the dsRNA expression levels in the GMP relevant for the FF safety assessment since they are not representative of those of the active siRNAs in a plant.

- For the FF safety assessment, it is noted that dietary silencing RNAs are generally rapidly degraded shortly after ingestion, unless chemical modifications increasing their stability are introduced, and face several cellular and intracellular barriers to their absorption. Therefore, the amount of absorbed dietary silencing RNAs can be considered negligible in humans and animals and limits the possibility to reach a tissue or functional location in sufficient amounts to exert any biological effect. Based on this, EFSA's GMO Panel considers that in general no dedicated animal studies on the safety of silencing RNAs are necessary.
- For the NTO risk assessment of pest/pathogen-resistant dsRNA-expressing GMPs for cultivation, it is agreed that bioinformatic analyses could identify NTOs that harbour genes with some level of sequence homology to the gene intended for silencing in the target pest/pathogen, and thus aid the selection of NTOs that require further consideration in the risk assessment (Devos et al., 2019a,b). However, at present, the presence of RNAi activity in NTOs cannot be reliably predicted in all representative NTOs through bioinformatic data. Therefore, this approach cannot be used as a stand-alone tool yet (Ramon et al., 2014; Roberts et al., 2015; Devos et al., 2019a,b). To make more reliable predictions, further research is needed to define the exact rules for small RNA-target matches, design suitable algorithms and increase knowledge on genomes and their expression, especially in non-model lines and other species (Ramon et al., 2014; Casacuberta et al., 2015).

Overall, the tiered-based strategy for NTO risk assessment can be used as outlined in EFSA Panel on Genetically Modified Organisms [EFSA GMO] (2010) and Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP] (2014). Laboratory bioassays are considered appropriate to assess RNAi effects on NTO fitness and performance. However, exposure parameters, the most sensitive endpoints to measure, and dose-response relationships for siRNA targets may need to be established for NTOs that are susceptible to RNAi, on a case-by-case basis (Federal Insecticide, Fungicide and Rodenticide Act [FIFRA], Scientific Advisory Panel [SAP], 2014; Devos et al., 2019a,b).

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NP conceived and took the lead in writing the manuscript. NP, YD, AL, and EW wrote sections of the manuscript. FÁ-A contributed tables and figures and formatted the manuscript. NP, YD, and EW provided critical feedback. All authors read and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Plant Science

REVIEW
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Safety Considerations for Humans and Other Vertebrates Regarding Agricultural Uses of Externally Applied RNA Molecules

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The potential of double-stranded RNAs (dsRNAs) for use as topical biopesticides in agriculture was recently discussed during an OECD (Organisation for Economic Co-operation and Development) Conference on RNA interference (RNAi)-based pesticides. Several topics were presented and these covered different aspects of RNAi technology, its application, and its potential effects on target and non-target organisms (including both mammals and non-mammals). This review presents information relating to RNAi mechanisms in vertebrates, the history of safe RNA consumption, the biological barriers that contribute to the safety of its consumption, and effects related to humans and other vertebrates as discussed during the conference. We also review literature related to vertebrates exposed to RNA molecules and further consider human health safety assessments of RNAi-based biopesticides. This includes possible routes of exposure other than the ingestion of potential residual material in food and water (such as dermal and inhalation exposures during application in the field), the implications of different types of formulations and RNA structures, and the possibility of non-specific effects such as the activation of the innate immune system or saturation of the RNAi machinery.

Keywords: double-stranded RNA, biopesticide, human safety, RNA interference, sprayable dsRNA

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INTRODUCTION AND PURPOSE OF THIS REVIEW PUBLICATION

The OECD (Organisation for Economic Co-operation and Development) Conference on RNA interference (RNAi)-based pesticides provided an overview of the current state of the art related to externally applied double-stranded RNA (dsRNA)-based products, also called exogenously or topically applied dsRNA. The purpose of the meeting was to facilitate exchanges between policymakers, academia, and industry on the implications of these products in health, the environment, and regulation, and to solicit inputs and recommendations based on these discussions[1]. The Conference was divided into three sessions. The first session provided a summary of the state-of-the-art of this technology: molecular mechanism and relevant RNAi pathways, current understanding of RNAi in different organisms, specificity level and its potential impact on non-target species, as well as the challenges associated with achieving RNAi efficacy in insects.

[1]https://www.oecd.org/chemicalsafety/pesticides-biocides/conference-on-rnai-based-pesticides.htm

The second session dealt with factors related to variation in insect responsiveness to environmental dsRNA, the environmental dissipation of dsRNA molecules in soil, water and plants, and the aspects of RNA-seq (RNA-sequencing) to validate RNAi data. It also presented an overview of available literature on the possible effects of exogenous dsRNA in humans and other vertebrates and addressed the regulatory experience with dsRNA applications in human therapeutics.

The third and final session summarized regulatory and risk assessment experiences whereby presenters identified "problem formulation" as a regulatory pathway similar to that for other biologically based active ingredients, explored better decision making ideas, and considered a case-by-case approach to assess ecological risks. It also reviewed the use of formulations to overcome hurdles for controlling insects that are recalcitrant to dsRNA and the experience of experts on the regulation of RNAi-based genetically engineered (GE) crops. To date, there is no precedent of externally applied RNAi-based products approved by regulatory agencies, even though the technology has already been developed and approved for genetically engineered (GE) crops. Although there are substantial differences between RNAi-based GE crops and biopesticides that impact the risk assessment of both products differently (such as dsRNA exposure duration, route, and dose), the experience of experts on regulation of RNAi-based GE crops provides helpful information on the safety of this new technology, guides interpretations of new studies, and supports regulatory requirements.

This report focuses on the third OECD meeting session that presented the possible effects of RNAi-based biopesticides in humans and other vertebrates. To introduce RNAi technology and its mode of action, the RNAi mechanism and main gene silencing pathways are described. RNAi technology comes as an alternative crop protection solution that enables a more specific, safer, and environmentally friendly tool for agricultural production (Bachman et al., 2013, 2016; Dubelman et al., 2014; Petrick et al., 2015; Parker et al., 2019). Even though RNAi technology has been studied for more than a decade and has been developed for use in GE crops (Baum et al., 2007; Bolognesi et al., 2012) and biopesticide products (Joga et al., 2016; McLoughlin et al., 2018), most of the available data concerning mammalian risk assessment comes from learnings within therapeutic research and studies developed to support the safety of GE crops. Because of its relevance to the topic and the ability to translate the data to RNAi-based biopesticides, this review presents and discusses key RNA studies in the GE crop literature and the history of safe consumption of dsRNA by humans and animals as well as describes the biological barriers responsible for the lack of response to exogenous RNA in these organisms. Additional considerations for human health specifically important for RNAi-based products were also discussed during the OECD Conference and are further discussed herein. The session included the implications of different types of formulations or forms of RNA structures, considered the other routes of exposure of a sprayable product during the product application (dermal and inhalation) and discussed the possibility of non-specific effects such as the activation of the innate immune system or RNAi machinery saturation.

OVERVIEW OF RNAi MECHANISMS IN VERTEBRATES

RNAi is a post-transcriptional process ubiquitous in eukaryotes that results in degradation or translational suppression of specific messenger RNA (mRNA) molecules, leading to a reduction in protein production. Suppression of the gene product occurs inside the cells and is initiated from either exogenous dsRNA or RNA molecules originating internally from the nucleus (reviewed by Carthew and Sontheimer, 2009). Herein, we discuss two key RNAi pathways that can be leveraged for gene expression regulation in an agricultural setting, small interfering RNA (siRNA) and microRNA (miRNA).

The siRNAs are short duplex sequences of ∼21–23 nucleotides (nt) (Elbashir et al., 2001) derived from the cleavage of both endogenous and exogenous dsRNAs. The dsRNA is specifically processed in the cellular cytoplasm by Dicer and Dicer-like proteins, which are members of the RNase III family of nucleases (Macrae et al., 2006). The duplex siRNAs are unwound by a helicase (yet to be identified) separating both sense and antisense strands (reviewed by Bartel, 2004). The siRNA strand with the less stable base pair at its 5′ end in the duplex, called the antisense strand, is loaded into a multiprotein complex called RISC (RNAi-Induced Silencing Complex) and the sense strand is degraded (reviewed by Winter et al., 2009). The antisense strand acts as a guide that recognizes the target mRNA by complementary base-pairing and Argonaute, a component of RISC, degrades the mRNA, leading to gene product suppression (Martinez et al., 2002; Khvorova et al., 2003; Winter et al., 2009).

The miRNAs are processed from their long dsRNA precursors into short ssRNA molecules that feed into an analogous pathway to that described above for siRNAs, leading to gene product suppression of complementary mRNA targets. This can occur through both translational suppression (Olsen and Ambros, 1999; Hutvágner and Zamore, 2002; Georgantas et al., 2007) and mRNA cleavage (Mansfield et al., 2004; Yekta et al., 2004). In animals, the miRNA precursors, termed primary miRNAs (pri-miRNAs) have a complex structure consisting of self-complementary sequences separated by a short non-complementary sequence. These molecules fold into intramolecular hairpins and often contain a small number of mismatched bases that create bubble-like structures (Cullen, 2004). The pri-miRNA is processed in the nucleus by Drosha, another enzyme member of the RNase III family, generating ∼65 nt stem-loop intermediate known as miRNA precursor (pre-miRNA) (Lee et al., 2003). The pre-miRNAs are transported to the cytoplasm through the nuclear export receptor Exportin-5 (Expo-5) and processed by Dicer, generating a ∼20 nt mature miRNA which is similar in both structure and function to the siRNA duplexes (Hutvágner and Zamore, 2002). Despite the similarity with siRNA duplexes, each miRNA hairpin precursor molecule produces a single miRNA duplex, whereas each long dsRNA produces multiple siRNAs. As with processed siRNAs,

miRNAs are unwound by helicases, the sense strand degraded, and the antisense strand incorporated into RISC (Khvorova et al., 2003). The miRNA guides RISC to the mRNA target and the complex and its component Argonaute induces mRNA degradation or translational repression (Meister and Tuschl, 2004). Different than the siRNA mechanism where all bases generally contribute to its target specificity, complementarity between a miRNA and its target is usually partial, meaning it can regulate transcripts with limited complementarity to the antisense strand of the miRNA duplex (Lam et al., 2015). However, the high complementarity with a contiguous stretch of at least six nucleotides beginning at position two of the 5′ end of the miRNA, the seed region, has shown to be important for miRNA induced gene regulation (Jackson et al., 2006). Pairing exclusively with the seed region is not enough to induce the target mRNA cleavage but may result in translational pause (Mullany et al., 2016).

HISTORY OF SAFE RNA CONSUMPTION

As presented above, RNAi is a highly conserved mechanism in eukaryotes for gene expression regulation. Small RNAs such as siRNAs and miRNAs are ubiquitous in commonly consumed plant and animal-derived foods. A number of small RNAs with perfect complementarity to human and animal genomes and transcriptomes have been identified in crops widely consumed globally, such as soybean, corn, and rice (Ivashuta et al., 2009). Corn specifically contains endogenous small RNAs that match approximately 450–2300 unique protein coding RNA transcripts in rat, mouse, and human (Petrick et al., 2016a). Fresh market fruits and vegetables also contain small RNAs with sequence complementarity to human genes. Most of these RNAs are likely derived from their genome, but a portion may also originate from plant viruses, which is an exogenous source (Frizzi et al., 2014). The established history of safe consumption of both exogenous and endogenous RNA molecules in food and feed that have 100% sequence complementarity to human and animal transcripts suggests that there is no negative biological effect of ingested RNAs, and supports safety of these molecules for use as agricultural active ingredients (Petrick et al., 2013; Frizzi et al., 2014).

In addition to the safe consumption of conventional crops, fruits, and vegetables, RNAi-mediated plant phenotypes have been found in many domesticated crops and have been used in approved biotech crop traits for more than two decades (reviewed by Petrick et al., 2013; Sherman et al., 2015). Some examples of RNAi-based traits include RNAi-mediated resistance to the ipomovirus CBSUV in cassava (Yadav et al., 2011), Innate™ potatoes with reduced acrylamide production potential and blackspot bruise resistance (Simplot, 2014) and recently approved by US EPA, MON 87411, a corn plant expressing the DvSnf7 RNA PIP (plant-incorporated protectant), encoding a dsRNA that confers RNAi-mediated control of corn rootworms (Bachman et al., 2016). In support of the human and mammalian safety assessment of MON 87411, Petrick et al. (2016a,b) performed a 28-day repeat dose toxicity study in mice with DvSnf7 RNA and did not observe any effects on body weights, food consumption, clinical observations, clinical chemistry, hematology, gross pathology, or histopathology endpoints. They concluded there are no adverse health effects in mammals administered an insect active RNA molecule at doses millions to billions of times higher than anticipated human exposures (Petrick et al., 2016a,b).

BIOLOGICAL BARRIERS

Vertebrates consume RNA molecules with every meal through foods of plant, animal, or fungal origin. This includes dsRNAs of various lengths that, based on sequence, would be capable of initiating the RNAi pathway if they were to reach a target cell. As presented previously, there are many such dietary dsRNAs that have sequence identity to genes in consuming vertebrates (Heisel et al., 2008; Ivashuta et al., 2009; Jensen et al., 2013; Frizzi et al., 2014; Petrick et al., 2016a) and without biological barriers protecting such organisms from these RNAs, every bite of every meal would present a potential source of regulation of protein production. Biological barriers faced by ingested RNAs (reviewed by O'Neill et al., 2011; Petrick et al., 2013) ensure homeostasis after RNA ingestion rather than a potential for these RNAs to impact gene expression in the consuming organism.

Following ingestion, food is chewed, and during this process, dietary RNA molecules are presented with nucleases in the saliva (Park et al., 2006) that begin to break down ingested RNA. As food passes the "oral phase" of digestion and is swallowed, dietary RNAs reach the stomach, which presents a digestive environment that results in extensive digestion of ingested nucleic acids (Petrick et al., 2013; Huang et al., 2018). This digestion occurs through a low pH environment in the stomach and leads to denaturation and depurination of nucleic acids followed by hydrolytic fragmentation (O'Neill et al., 2011). From the stomach, RNAs in partially digested food reach the small intestine, containing a digestive milieu that includes nucleases and degradative enzymes secreted from the pancreas that degrade nucleic acids, further digesting ingested RNA molecules into shorter nucleotides (O'Neill et al., 2011). From the small intestine, RNAs can transit through the gastrointestinal tract and be eliminated in the feces or in some cases smaller nucleic acids may be absorbed into the gastrointestinal epithelium and undergo further distribution throughout the body.

For an exogenous RNA to undergo absorption from the lumen of the intestinal tract, the RNA must cross a series of cellular membrane barriers. This includes the apical and distal membranes of the gastrointestinal (GI) epithelial cells that a given RNA must transverse and if such an RNA is to reach the bloodstream, both apical and distal membranes of the vascular endothelium. This must also occur for an RNA to cross into a distal tissue, e.g., the RNA would have to again leave the vasculature through the endothelium and cross the epithelium of another tissue to have the potential to regulate gene expression in that tissue. Each of these cellular membrane layers is a lipid bilayer that is highly impermeable to polar macromolecules such as RNA (Gilmore et al., 2004;

Petrick et al., 2013). Any RNA reaching the interior of a cell must also escape endosomes, cellular compartments that sequester a majority of RNA molecules that enter a cell (e.g., 98–99%), posing a significant barrier to efficacy of RNA therapeutics (White, 2008; Gilleron et al., 2013). This would also present a significant barrier to any ingested RNAs that undergo cellular uptake. To further complicate the transit of dietary RNA through the systemic circulation, nucleases in the blood serve to degrade RNA molecules (Houck, 1958; Layzer et al., 2004; White, 2008; Christensen et al., 2013). In addition, RNA is cleared rapidly from the bloodstream via renal elimination (White, 2008; Molitoris et al., 2009; Thompson et al., 2012). These barriers are reviewed and pictorially represented in the peer reviewed scientific literature (Petrick et al., 2013). The collective series of barriers described above and summarized in **Table 1** presents a formidable challenge to therapeutic developers, necessitating chemical stabilization of RNA therapeutics and their formulation in lipid and other delivery systems to enable escape from these barriers and to increase their resistance to degradation (O'Neill et al., 2011; Forbes and Peppas, 2012).

The efficacy of these barriers can be observed in therapeutic studies in which injected RNA drugs that are unformulated are both readily degraded and rapidly excreted (Tillman et al., 2008; White, 2008; Molitoris et al., 2009; Thompson et al., 2012; Christensen et al., 2013). Observations of limited RNA uptake due to biological barriers have been made with ingested/orally administered RNA molecules in both dietary and therapeutic settings (Tillman et al., 2008; Dickinson et al., 2013; Snow et al., 2013; Huang et al., 2018), a subject that will be discussed in greater detail within this manuscript.

PUBLISHED MAMMALIAN STUDIES

The above discussion on biological barriers to exogenous RNA molecules emphasizes the oral route of exposure as the relevant route of exposure to RNAs in foods, including those that confer traits to GE crops. The oral route is also of key importance to the potential impact of any RNA residues in foods resulting from topical uses of nucleic acids in an agricultural setting. Based on the history of safe consumption of RNA molecules in the diet (including dsRNAs with sequence identity to the consuming organism) and the biological barriers detailed above, the weight of the evidence suggests that ingested nucleic acids are neither absorbed to a significant extent nor capable of triggering a biological response in a consuming organism. This is largely due to the collective impact of each of the individual biological barriers discussed above, leading to a significant reduction in dsRNA levels in terms of the amount available for possible functional activity relative to the amount ingested; these barriers result in insufficient copies being available within a given cell to mediate biological function (White, 2008; Snow et al., 2013; Witwer, 2016). Plant miRNA uptake in mice is quite limited (i.e., less than one copy per ten cells) and more importantly, is insufficient for mediating RNAi in the ingesting organism, which requires at least 100 copies of RNA per cell (Snow et al., 2013). The number of RNA copies per cell needed to mediate biologically meaningful RNAi may be as many as 1,000–10,000 copies (Title et al., 2015). Plant small RNAs are also bound tightly to Argonaute proteins within the RISC complex as necessary for their function and they are not known to either freely dissociate from these complexes or undergo uptake and exchange (either free or complexed) into functional host Argonautes in order to engage targets in a consuming organism (Witwer, 2016). Therefore, these barriers and limited uptake along with the inability of exogenous plant RNAs to function in a consuming organism severely limit the possibility of diet-derived small RNAs having activity in the ingesting organism.

TABLE 1 | List of significant biological barriers to ingested RNAs in humans.

Biological Barriers		
Gastrointestinal	**Systemic**	**Cellular**
Saliva	Vascular endothelium	Cellular and nuclear membrane barriers
Stomach acids/Digestive enzymes	Serum nucleases	Endosomes and lysosomes
Pancreatic nucleases	Renal filtration and elimination	Sufficient sequence identity
GI epithelium and tight junctions	Tissue epithelium	Gene target accessibility

The concept of potential uptake and activity of dietary small RNAs was evaluated in the context of rice miRNAs. In a 2012 peer-reviewed publication, it was suggested that a specific miRNA in rice (miR168a) was absorbed into the bloodstream of ingesting mice and into systemic tissues where it reduced levels of a targeted protein (LDL Receptor Adaptor Protein 1, LDLRAP1) and mediated downstream physiological impacts on cholesterol levels (Zhang et al., 2012). Possible explanations for the findings of Zhang et al. (2012) include laboratory contamination and very low level detection, leading to false positives within PCR-based measurements of RNA uptake (Witwer et al., 2013; Lusk, 2014; Tosar et al., 2014) and from issues surrounding the experimental design of the feeding studies, e.g., diets were not nutritionally balanced (Dickinson et al., 2013). After a 12-h fast, Zhang et al. (2012) fed mice a carbohydrate rich diet of 100% raw rice (contains miR168a) for several days prior to observing dysregulation of a cholesterol related protein and serum LDL cholesterol levels. When diets abundant in miR168a were fed to mice following a 2-week washout period of feeding on a synthetic diet (no plant material or rice-derived miR168a), the same serum cholesterol impacts of diet feeding were observed as those of Zhang et al. (2012), but only when nutritional equivalence of the test and control feeding regimen was not maintained (e.g., only when a diet of mostly rice was given) (Dickinson et al., 2013). Such differences in cholesterol were not observed when a miR168a-rich but nutritionally balanced diet was administered following the washout period. No apparent absorption of miR168a was observed in the blood or tissues of mice in this feeding study (Dickinson et al., 2013). No modification of the LDLRAP1 target protein expression levels were observed by Dickinson et al. (2013) under any experimental conditions using a mouse-specific ELISA assay run at several

dilutions, indicating that Western blotting conducted by Zhang et al. (2012) may not have accurately reflected impacts of dietary miRNAs on protein levels. Therefore, dietary miR168a does not undergo absorption to a biologically meaningful extent following rice feeding and any levels of absorption are insufficient to modulate gene expression levels in the consuming mammal.

When droplet digital PCR was leveraged to evaluate miRNA uptake from feeding experiments in rhesus monkeys, uptake from the diet was not evident over a time course (Witwer et al., 2013). This PCR method allows for many PCR reactions that collectively result in identification of false-positive amplifications. Subsequent publications have demonstrated that contamination of PCR reactions can lead to false-positive amplifications within biological samples (Tosar et al., 2014). An elegant study using knockout mice for specific miRNAs demonstrated that mice do not take up dietary miRNAs in sufficient quantities to mediate gene suppression (e.g., less than one copy per cell detected), as evaluated through feeding these same miRNAs to knockout mice (Snow et al., 2013). This study by Snow and colleagues also conducted fruit feeding studies in healthy humans and were unable to detect fruit-derived miRNAs after measuring them in fruit and looking for them in blood following consumption. This calls into question the detection of exogenous RNAs in the bloodstream of humans and other mammals in laboratory and sequencing studies (Wang et al., 2012; Zhang et al., 2012; Tosar et al., 2014; Bagci and Allmer, 2016; Kang et al., 2017) and the role of contamination in these analyses, as detected miRNAs have included sequences derived from microbes, yeast, insects, worms, rodents, and foods for which plausibility of exposure is not apparent (i.e., rat miRNAs in human samples; carrot, cabbage, and sorghum miRNAs in mouse samples). Microbial sequences in the bloodstream could indicate sepsis and the presence of exotic RNAs in the bloodstream from the diet seems implausible with both being indicative of contamination. This has been extensively evaluated in 800 human data sets (Kang et al., 2017), supporting the conclusion that contamination and not dietary uptake is the most plausible explanation for widespread and/or abundant detection of exogenous miRNAs in mammalian blood samples.

Despite substantial evidence from well-controlled feeding studies in rodents and humans that calls into question the ability for RNA from the diet to undergo significant uptake and have putative activity in mammals (Dickinson et al., 2013; Snow et al., 2013; Witwer et al., 2013), the dietary RNA uptake hypothesis has continued to be explored. This research has resulted in a number of feeding studies claiming RNA absorption from the diet through various plant sources (Liang et al., 2015; Yang et al., 2015a, 2016, 2017) and potential physiological impacts including papers claiming the ability of dietary RNAs to treat highly infectious viruses (Zhou et al., 2015), regulate intestinal growth (Li et al., 2019), and treat cancer (Mlotshwa et al., 2015). Some experimental issues with these studies (as described in detail by Petrick et al., 2016a) call into question whether these papers indeed indicate the ability of typical dietary RNAs (e.g., miRNAs) to impact gene expression in a consumer (Petrick et al., 2016a). For example, Zhou et al. (2015) and several of the above papers by Yang and colleagues evaluated putative biological impacts of a highly thermostable and GC-rich (85% GC content) ribosomal RNA fragment termed "MIR2911" (other RNAs in the fed fraction were degraded by heat during preparation), which is not a miRNA and its properties are not reflective of a typical dietary miRNA. Furthermore, this RNA was reported by Zhou and colleagues to have potent antiviral activity (reduction in viral titer, reduction in body weight loss, and increased survival) despite its low level in the diet. These data contradict years of pharmaceutical research regarding lack of oral RNA efficacy and claims of biological activity of orally administered/ingested RNAs have been presented in only a small number of papers to date (Zhang et al., 2012; Mlotshwa et al., 2015; Zhou et al., 2015; Li et al., 2019), with these reports remaining unconfirmed.

Li et al. (2019) noted that after feeding corn containing diets to mice, an RNAi-mediated mechanism impacts several genes and their targeted proteins. However, the *in vivo* data demonstrate modest changes in relative protein levels of approximately 1–3 fold (by Western blot with anti-human antibodies) in a small sample size ($n = 4$) at a single time point (7 days). Without a deeper understanding of the comparative nutritional components of the fed diets (e.g., comparison of fat, protein, carbohydrates, and key nutrients across the diets) and evaluation of the normal range of expression variability of the evaluated target proteins, along with thorough histopathological assessment of these animal intestines, the physiological relevance of the slight changes in mRNA and protein levels and the noted changes in morphology following corn feeding is difficult to assess. Furthermore, the results of this paper are inconsistent with results demonstrating no uptake of corn miRNAs into the bloodstream of mice after 2 weeks of oral dosing (Huang et al., 2018).

The hypothesis that "you are what you eat" and that nutrition may serve as a therapeutic modality is an attractive one. This may explain in part why there have been many reviews on the topic of dietary miRNA uptake and/or activity in mammals, often with a theme of dietary miRNAs being potential mediators of our responses to foods and also reviews that challenge this concept (Cottrill and Chan, 2014; Witwer and Hirschi, 2014; Hirschi et al., 2015; Yang et al., 2015b,c). The result of these reviews has been a great deal of interest in the subject, however, the primary literature leveraged in these reviews lacks robust evidence that any of the reported uptake and activity of dietary miRNAs results in physiologically meaningful impact to the consuming organism or adverse impact to animals following consumption.

There have been two published 28-day repeated-dose oral toxicology studies looking at high doses of insecticidal double-stranded RNA sequences fed to mice (Petrick et al., 2015, 2016a,b). Following 28-days of repeat oral dosing of mice at doses of $\geq$48 mg/kg body weight with siRNAs or a long dsRNA with 100% sequence complementarity to mouse vacuolar ATPase (gene target provides corn rootworm control when rootworm sequence is expressed in corn), no treatment-related toxicity or target gene suppression was observed. When a corn rootworm active RNA sequence (240 base pair active dsRNA embedded in a 968 nucleotide RNA) was fed to mice at doses of up to 100 mg/kg body weight, no treatment-related effects were observed (Petrick et al., 2016a). Therefore, the no-observed adverse effect level was 100 mg/kg body weight (the highest dose tested), a dose that

is estimated to be at least 2.5 billion times higher than mean per capita maize consumption in Europe and the United States (Petrick et al., 2016a,b). Based on the weight of the evidence from mammalian toxicology studies, ingested RNA molecules do not undergo physiologically meaningful uptake and do not present a hazard to humans following ingestion.

Potential for uptake and impacts of exogenous dsRNAs following ingestion have been considered by regulatory authorities. The European Food Safety Authority (EFSA) noted that, "Based on the current knowledge, gained in pharmaceutical research and development, RNAi molecules show limited bioavailability, quick turn-over (for further reading please refer, for example, to Ballarín-González et al., 2013) and no adverse effects following oral gavage (even for formulations specifically designed to maximize their effects)." (Ballarín-González et al., 2013; EFSA, 2014). A Scientific Advisory Panel held by the US EPA (USEPA, 2016) noted that, "there are no reliable evidence [*sic*] that exogenous dsRNAs are taken up from the gut into mammalian circulation to exert its functions in the ingesting organism." Furthermore, the panel considered such impacts unlikely due to arguments concerning stoichiometry, noting the low levels of blood concentration relative to those needed to induce regulation of gene expression. Food Safety Australia New Zealand considered this topic and concluded that, "A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of these RNAs from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect (FSANZ, 2015)." Based on the weight of the evidence from mammalian studies and regulatory considerations, ingestion of RNA molecules does not present a hazard to humans or other mammals.

PUBLISHED STUDIES IN NON-MAMMALIAN VERTEBRATES

It still remains unknown whether all the factors required to initiate RNAi from ingestion of exogenous dsRNAs exist in non-mammalian vertebrates. Sifuentes-Romero et al. (2011) reviewed several studies of the RNAi response in a variety of animals, including frogs (*Xenopus laevis*), fish (*Danio rerio*, *Oncorhynchus mykiss*, *Cyprinus carpio*), chicken (*Gallus gallus*), and turtle (*Trachemys scripta*) and concluded that there is enough evidence to support an effective, potent, and reproducible RNAi response in these organisms using invasive delivery techniques, such as microinjections and electroporation. Similar responses could also be observed in birds (*Zonotrichia leucophrys gambelli*) when siRNAs were directly administrated into their brains (Ubuka et al., 2012). In Sea lampreys (*Petromyzon marinus*), gene suppression and phenotype effects were observed via injection of siRNA into embryos. Larvae of lampreys also showed gene suppression after feeding on a siRNA with a liposome-based formulation as the transfection reagent, however, feeding naked siRNA (without transfection reagents) failed to induce a response (Heath et al., 2014). These data all confirm the feasibility of RNAi technology as a tool for conducting fundamental biological process studies and loss-of-function experiments in a variety of organisms and not only model systems. However, to date, there is a lack of evidence of RNAi effects in vertebrates orally exposed to naked dsRNA. Bachman et al. (2016) carried out an extensive study on the effects of dsRNA on several organisms as part of the ecological risk assessment for DvSnf7, a dsRNA-based PIP (Bachman et al., 2016). Included in the study, a corn rootworm active dsRNA (1000 μg dsDvSnf7/kg diet) was incorporated into the diet of Bobwhite quail (*Colinus virginianus*) and the animals were observed for 14 days. Body weights, signs of toxicity, abnormal behavior, and mortality were recorded, and no adverse effects were observed, indicating an absence of non-specific RNAi responses (Bachman et al., 2016).

HUMAN HEALTH RISK CONSIDERATIONS FOR RNA-BASED BIOPESTICIDES

Pesticides are an indispensable tool for farmers and are used as an efficient and beneficial tool for pest management in most sectors of agricultural production. However, there are always hazards and associated risks associated with the exposure of farmers and/or professional applicators when mixing and applying the product or working in treated fields (Damalas and Koutroubas, 2016), and for the general public in the case of residues in food and drinking water (Damalas and Eleftherohorinos, 2011). The risk of a pesticide to any living organism is assessed by estimating its associated hazards or potential to cause harm (due to the inherent toxicity of a particular substance) and the possibility of exposure (Damalas and Koutroubas, 2016). When exposure occurs, both the exposure amount (dose) and duration (length and frequency) are important in understanding potential risks associated with pesticide toxicity (Frank and Ottoboni, 2011). Therefore, risk assessment is a comprehensive evaluation of the pesticide toxicity profile and an assessment of exposure. Pesticide labels contain not only information regarding the potential hazards of the product but also use requirements that reduce potential exposures. When agricultural chemical products are used in accordance with the label instructions, even toxic substances can be applied with relatively low risk.

Other Routes of Exposure

Exposure is required in order for any risk to exist. For humans, there are several different possible routes of exposure to dsRNA-biopesticides. Accidental oral exposure or residues in food and water represent two possible scenarios for ingestion and the potential risks associated with these exposures has been discussed above. Other possible exposure routes to consider are through dermal absorption and inhalation, potentially relevant routes for occupational exposure (Maroni et al., 1999).

Dermal Absorption

It is appropriate to consider potential dermal exposure to agricultural products that may be deployed in or applied to the

field. In the case of dsRNA molecules, the scientific literature demonstrates that they undergo limited dermal uptake. Nucleic acids are potential therapeutics for various diseases due to their specificity, and delivery through the topical route would be desirable for drug developers. However, there are well known delivery challenges for these therapeutics due to biological barriers such as nuclease degradation, rapid clearance from the bloodstream, and poor bioavailability- barriers that remain challenges for systemically delivered nucleic acid therapeutics (Rayburn and Zhang, 2008; Pecot et al., 2011). Several challenges exist for topical delivery of nucleic acids despite advantages over intravenous and oral delivery. For example, topical delivery avoids enzymatic degradation in the bloodstream, lowers systemic toxicity potential and provides sustained and controlled delivery (Brown et al., 2006). A formidable barrier to absorption is posed by the stratum corneum, a thin layer of anucleated corneocytes held tightly together by a lipid matrix that forms the outermost layer of skin, and serves primarily as a barrier to foreign materials (Zakrewsky et al., 2015). If foreign materials are to cross the stratum corneum, this must occur either through diffusion via lipid channels and/or transcellular passage through corneocytes, or via entry through sweat ducts or hair follicles (Zakrewsky et al., 2015). Transport within the lipid bilayers is the most common of these routes; however, this route excludes most foreign materials. Large hydrophilic molecules (short dsRNAs are >10 kD) undergo negligible transport across the skin without transport enhancers or various cellular membrane disruption techniques (e.g., microporation or electroporation) (Zakrewsky et al., 2015). Even in the very unlikely scenario of significant dsRNA absorption and systemic distribution following topical exposure, such RNAs would be expected to undergo rapid metabolism and clearance and would be subjected to the numerous biological barriers discussed above. Therefore, rapid breakdown and clearance, along with various dermal barriers to macromolecules (e.g., exogenous dsRNAs) greatly limit the potential for dermal toxicity of dsRNA molecules.

Inhalation

Most RNAi-based biopesticide products will be applied using similar methods as traditional chemical pesticides (e.g., spray applications), therefore, respiratory exposure should be considered as another potential exposure route. During a pesticide spray application, a significant portion of the product may not reach the intended target and may be transported to other areas through spray drift (van den Berg et al., 1999; Degrendele et al., 2016), which can potentially be inhaled and deposited in the human respiratory system. However, the pulmonary deposition of particles in the lung is dependent on the aerodynamic diameter of the inhaled droplets (Hinds, 1982). Most agricultural particles are large enough that they are not deposited in the lung, but rather, are cleared from the upper respiratory tract, resulting in a secondary oral exposure rather than pulmonary exposure (Sherman et al., 2015).

Challenges with the development of inhaled RNA therapeutics demonstrate how challenging it is to use this route to effectively deliver RNA molecules in humans. Some recent inhaled RNA therapeutic studies have shown advances in the delivery of RNAi-based drugs to the lungs (reviewed by Youngren-Ortiz et al., 2017; Thanki et al., 2018). However, a common conclusion across these studies is the requirement of the design and development of specifically engineered formulations to safely and effectively deliver the RNAi-based drug. The engineered molecules must overcome the existing biological barriers, such as degradation by RNase, mucociliary clearance, clearance by impaction and coughing, and alveolar macrophage clearance (Youngren-Ortiz et al., 2017). Adding to that, there is the challenge of developing suitable devices for pulmonary administration of inhalable RNAi-based therapeutic formulations (Thanki et al., 2018).

As with any pesticide, when spraying, one must take routine precautions to prevent inhalation of dsRNAs by applicators, for example, through the use of appropriate personal protective equipment (PPE). At present, there seems to be an absence of published data concerning the potential biological impact of inhalation of RNA molecules. Given that these can potentially be immunostimulatory molecules (via non-oral routes) per the literature, a non-sequence specific inflammatory response may occur upon significant exposures, hence the recommendation to use the appropriate PPE to limit inhalation exposures for spray applications as with any other sprayed crop protection product.

Different RNA Structures and Formulations

It is widely known that different species respond differently to environmental dsRNA (Rodrigues and Figueira, 2016; Wang et al., 2016; Christiaens et al., 2018a). In insects, for instance, lepidopterans are considered recalcitrant to naked dsRNA via oral delivery (Terenius et al., 2011; Shukla et al., 2016; Guan et al., 2018). In order to apply RNAi technology to manage non-responsive pests, several companies have developed different RNA structures as carriers to improve the delivery of dsRNA. In addition, because of its intrinsic structure, RNA forms can also be naturally modified to form several structures, such as supercoiled or in a hairpin. However, all forms of RNA structures, whether modified to increase the responsiveness of the target pest or occurring naturally, are likely to be degraded through the digestive process by the combination of RNases and acids found in the human digestive system (USEPA, 2014).

Another approach to enhance RNAi response in some pests is the development of formulations (Christiaens et al., 2018b; Dhandapani et al., 2019) aiming to improve delivery and availability of the dsRNA to the target. However, considering the complexity and the multiple biological barriers present in mammals (already discussed herein), it is unlikely that formulated RNAi-based products developed for agricultural uses will efficiently deliver dsRNA into human cells following ingestion. In support of this, researchers have developed several formulations to address the delivery and biostability of RNAi inside the human body and clinically relevant responses have been limited to injected drugs, such as vaccines and cancer therapy drugs (Ji et al., 2011; Kang et al., 2011; Lin et al., 2013; Cavallaro et al., 2017). O'Driscoll et al. (2019) reviewed the progress and feasibility of oral delivery of RNA-based drugs and have observed a lack of clinical trial data; indicating,

"while progress has been made through innovative formulation strategies to date clinical translation of oral products has not been realized" (O'Driscoll et al., 2019).

The wide variety of substances and technologies that can be developed and optimized into a cost-effective formulation to enhance dsRNA delivery and stability in the field can be a reality in the future. The recommendation raised during the OECD meeting to mitigate this risk is to consider each new formulation individually in a case-by-case assessment.

Non-specific Effects (Innate Immune System or RNAi Machinery Saturation)

Exogenous dsRNAs are known to stimulate the innate immune response (e.g., the interferon pathway) under experimental conditions permitting high levels of RNA exposure (Judge et al., 2005; Robbins et al., 2009; Jackson and Linsley, 2010). As reviewed by Petrick et al. (2013), such induction of the innate immune system has been characterized using *in vitro* systems that leverage transfection reagents and high RNA concentrations, and in some cases, in animal models. These responses are mediated via receptors that interact with dsRNA such as the Toll-like receptors (TLR3, TLR7, TLR8), and enzymes including the dsRNA binding protein kinase PKR, and the RIG-I and MDA-5 RNA helicases (Robbins et al., 2009). In animals, this induction of the innate immune response appears to be constrained to injection/systemic exposure to RNAs (Robbins et al., 2009) and there is no apparent evidence in the literature indicating that this occurs following oral exposure, the most relevant human exposure route for risk assessment considerations of agricultural uses of externally applied dsRNA.

In a set of mouse and blood cell studies from the pharmaceutical industry, sequence-dependent stimulation of the innate immune response in mice was demonstrated following intravenous delivery of dsRNAs (siRNAs) encapsulated in liposomes (Judge et al., 2005). However, this response did not occur with naked RNAs. Judge and colleagues note that delivery vehicles were required for immunostimulation and that encapsulated RNA formulations are protected from nucleases yielding extended circulation times relative to those not encapsulated. This dependency on delivery vehicles may stem from their ability to deliver RNA to the endosomal compartment of the cell that houses RNA-sensing pattern recognition receptors that facilitate an immune response (Robbins et al., 2009; Jackson and Linsley, 2010). Induction of the innate immune system was not observed with the injection of unmodified naked siRNAs (Heidel et al., 2004; Judge et al., 2005). An oral siRNA study using a specialized delivery vehicle to promote absorption did not show evidence of immunostimulation even in the presence of target gene suppression (Aouadi et al., 2009). Therefore, it is apparent that immunostimulation by dsRNAs appears to require specific delivery routes (e.g., injection), delivery vehicles, specific sequence motifs, stabilizing modifications, and significant exposures, all of which have limited or no relevance to dietary exposures that may occur from agricultural uses of dsRNA.

Although a review article has indicated immunostimulation as a possible hazard from oral exposure to dsRNAs in agriculture (in this case, from GE crops; Lundgren and Duan, 2013), exposure scenarios required for observation of immunostimulation (e.g., injection of high doses of nucleic acids) are not relevant to environmental or dietary exposures that could be encountered by humans or non-target mammals through exposure to exogenous RNAs. This is because immunostimulation from an ingested RNA would require absorption of a sufficient concentration for induction of the response, a phenomenon that is improbable given the multitude of biological barriers to the attainment of significant levels of systemic RNA after dietary consumption. DvSnf7 RNA, a corn rootworm active RNA molecule when expressed in corn plants (includes a 240 bp insect active dsRNA) was safely administered to mice via oral gavage for 28 days without any apparent clinical or toxicological signs of immunostimulation or immune response at a dose of up to 100 mg/kg (Petrick et al., 2016a). This dose is 2000 times higher than the 0.05 mg/kg cited as capable of inducing potent cytokine responses following dsRNA injection into the mouse (Judge et al., 2005). Furthermore, this 100 mg/kg dose is 50 times higher than the 2 mg/kg experimental intravenous siRNA dose used by Judge and colleagues that produced immunostimulation for formulated siRNAs but not naked siRNAs. The lack of oral immunostimulation by dsRNAs is further evidenced by the extensive history of safe consumption of RNAs in the diet from food, be they short or long dsRNAs (Fukuhara et al., 2006; Ivashuta et al., 2009; Jensen et al., 2013; Petrick et al., 2013; Frizzi et al., 2014), owing to the lack of appreciable absorption or systemic tissue exposures following ingestion.

Another putative risk from dsRNA in GE crops mentioned by Lundgren and Duan (2013) is the saturation of the RNAi machinery. In one of the seminal papers on RNAi machinery saturation, this phenomenon occurs *in vitro* in a dose-dependent manner after transfection of relatively high doses of small RNAs into cultured cells (Khan et al., 2009), exposure conditions that are not relevant to environmental or dietary exposures to exogenous dsRNAs. This phenomenon occurs as a result of a limited number of RISC complexes being overwhelmed by the amount of externally applied small RNA, under supraphysiological conditions. As reviewed by Jackson and Linsley (2010), other papers demonstrating RNA machinery saturation relied on "sustained high-level expression of [short hairpin] shRNAs in the liver of adult mice," a transgenic approach relying on over-expression of a short RNA hairpin in a mouse. This phenomenon was also reviewed by Grimm (2011). In contrast to these saturating doses, another study looking at *in vivo* delivery of exogenous RNAs achieved about 80% silencing of targeted transcript without affecting cellular miRNA biogenesis or function, e.g., this silencing did not result in saturation of the RNA machinery (John et al., 2007). Indeed, Lundgren and Duan do not seem convinced of the biological relevance of RNAi machinery saturation as their review stated that, "it is unclear how dsRNAs produced by plants could affect the RNAi machinery used by both target and non-target organisms and whether there will be sufficient small RNA produced by GM plants to saturate an organism's cellular machinery (Lundgren and Duan, 2013)." Questions on sufficiency of exposure are equally applicable to externally applied "topical dsRNA" due to limited levels of application in the field (i.e., applications to be

limited by cost of goods) and extensive barriers to exogenous dsRNAs in non-target mammals. Therefore, the plausibility of systemic dsRNA exposure from agricultural applications at levels capable of initiating RNAi machinery saturation is highly questionable. Toxicological studies in mammals (discussed above) exhibited no adverse findings at oral dose levels up to billions of times anticipated human dietary exposure, providing strong support for the implausibility of RNAi machinery saturation as a potential source of adverse effects from exogenous dsRNA exposures in mammals.

CONCLUSION

The data available indicate that significant systemic absorption of intact dsRNA following dietary exposure of RNAi-based biopesticides is highly improbable in humans and other vertebrates. The longstanding history of safe consumption of dsRNAs naturally found in all foods and feeds, including those with complementarity to human and vertebrate transcripts, supports the safety of these molecules for use as biopesticides. The principal reason for the lack of biological response to exogenous dsRNAs is the presence of multiple biological barriers at the gastrointestinal, bloodstream, and cellular levels in mammals. Even in the very unlikely scenario of significant dsRNA absorption and systemic distribution following exogenous exposure during the product application, such RNAs would be expected to undergo rapid metabolism and clearance. Owing in part to the history of safe consumption and the favorable toxicity profile of exogenous dsRNA molecules in mammals (including insecticidal sequences), biological barriers, and their fate *in vivo*, these biological macromolecules should not be presumed to be inherently more risky than conventional small molecule agrochemicals. Regulatory authorities have not yet established standard procedures for assessing dsRNA-based agricultural products. The safety assessment of each of these products is currently considered on a case-by-case basis by these authorities. However, the existing robust regulatory framework for small molecule agrochemicals is applicable as a general framework for conducting risk assessment of dsRNA-based agricultural products. As with any emerging technology, the regulatory framework will continue to evolve; however, the experience with the review of dsRNA-based GE crops has demonstrated that the existing regulatory paradigm for biologically based crop protection products is adequate for this mode of action. The OECD Conference, along with this paper, increase clarity on both hazard identification and potential risks of RNAi-based biopesticides while also promoting important dialogues among different stakeholders to help facilitate the exchange of ideas between them.

AUTHOR CONTRIBUTIONS

TR and JP contributed equally to draft the work and revised it critically for important intellectual content.

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Conflict of Interest: The authors of this publication work for GreenLight Biosciences, Inc. and Bayer Crop Science and conduct research in developing products based on topical RNAi.

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RNA-Spray-Mediated Silencing of *Fusarium graminearum AGO* and *DCL* Genes Improve Barley Disease Resistance

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Over the last decade, several studies have revealed the enormous potential of RNA-silencing strategies as a potential alternative to conventional pesticides for plant protection. We have previously shown that targeted gene silencing mediated by an *in planta* expression of non-coding inhibitory double-stranded RNAs (dsRNAs) can protect host plants against various diseases with unprecedented efficiency. In addition to the generation of RNA-silencing (RNAi) signals *in planta*, plants can be protected from pathogens, and pests by spray-applied RNA-based biopesticides. Despite the striking efficiency of RNA-silencing-based technologies holds for agriculture, the molecular mechanisms underlying spray-induced gene silencing (SIGS) strategies are virtually unresolved, a requirement for successful future application in the field. Based on our previous work, we predict that the molecular mechanism of SIGS is controlled by the fungal-silencing machinery. In this study, we used SIGS to compare the silencing efficiencies of computationally-designed vs. manually-designed dsRNA constructs targeting *ARGONAUTE* and *DICER* genes of *Fusarium graminearum* (*Fg*). We found that targeting key components of the fungal RNAi machinery via SIGS could protect barley leaves from *Fg* infection and that the manual design of dsRNAs resulted in higher gene-silencing efficiencies than the tool-based design. Moreover, our results indicate the possibility of cross-kingdom RNA silencing in the *Fg*-barley interaction, a phenomenon in which sRNAs operate as effector molecules to induce gene silencing between species from different kingdoms, such as a plant host and their interacting pathogens.

Keywords: RNA spraying, RNA silencing, spray-induced gene silencing, *Fusarium graminearum*, AGO and DCL

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INTRODUCTION

Diseases of cereal crops, such as Fusarium head blight caused by phytopathogenic fungi of the genus *Fusarium* and primarily by the ascomycete *Fusarium graminearum* (*Fg*), exert great economic and agronomic impacts on global grain production and the grain industry (Goswami and Kistler, 2004; Kazan et al., 2012; McMullen et al., 2012). In addition to significant yield losses, food quality is adversely affected by grain contamination with mycotoxins, representing a serious threat

to human and animal health (Ismaiel and Papenbrock, 2015). Plant-protection and toxin-reduction strategies are presently mediated by chemical treatments. Currently, the application of systemic fungicides, such as sterol demethylation inhibitors (DMIs), is essential for controlling Fusarium diseases and to assist in reaching the maximum attainable production level of high-yield cultivars. DMI fungicides act as ergosterol biosynthesis inhibitors because of cytochrome P450 lanosterol C-14α-demethylase (CYP51) binding, which subsequently disturbs fungal membrane integrity (Kuck et al., 2012). Because of a shortage of alternative chemicals, DMIs have been used extensively in the field since their discovery in the 1970s. Therefore, it is hardly surprising that reduced sensitivity, or even resistance to DMI fungicides, has begun to develop in many plant pathogenic fungi (Yin et al., 2009; Spolti et al., 2014). These alarming developments demonstrate that novel strategies in pathogen and pest control are urgently needed.

RNAi is known as a conserved and integral part of the gene regulation processes present in all eukaryotes and is mediated by small RNAs (sRNAs) that direct gene silencing at the post-transcriptional level. Post-transcriptional gene silencing (PTGS) starts with the initial processing or cleavage of a precursor double-stranded (ds)RNA into short 21–24 nucleotide (nt) small-interfering RNA (siRNA) duplexes by an RNaseIII-like enzyme called Dicer (Baulcombe, 2004; Ketting, 2011). Double-stranded siRNAs are incorporated into an RNA-induced silencing complex (RISC) that initially unwinds the siRNA, thereby generating an antisense (or guide) strand which base-pairs with complementary mRNA target sequences. Subsequent degradation of the targeted mRNA mediated by an RNase protein called Argonaute (AGO) prevents translation of the target transcript (Vaucheret et al., 2004; Borges and Martienssen, 2015) ideally resulting in a loss of function phenotype. Therefore, RNAi has emerged as a powerful genetic tool not only in fundamental research for the assessment of gene function but also in various fields of applied research, such as agriculture. In plants, RNAi strategies have the potential to protect host plants against infection by pathogens or predation by pests mediated by lethal RNAi signals generated *in planta,* a strategy known as 'host-induced gene silencing' (HIGS; Nowara et al., 2010) (for review, see Koch and Kogel, 2014; Yin and Hulbert, 2015; Guo et al., 2016; Zhang et al., 2017; Gaffar and Koch, 2019; Qi et al., 2019). In addition to the generation of RNA-silencing signals *in planta*, plants can be protected from pathogens and pests by spray-applied RNA biopesticides designated as spray-induced gene silencing (SIGS) (Koch et al., 2016; Wang et al., 2016; Konakalla et al., 2016; Mitter et al., 2017a; Kaldis et al., 2018; Koch et al., 2019). Regardless of how target-specific inhibitory RNAs are applied (i.e., endogenously or exogenously), the use of HIGS and SIGS technologies to control *Fusarium* species have been shown to be a potential alternative to conventional pesticides (Koch et al., 2013; Ghag et al., 2014; Cheng et al., 2015; Hu et al., 2015; Chen et al., 2016; Pareek and Rajam, 2017; Bharti et al., 2017; Baldwin et al., 2018; Koch et al., 2018, 2019) supporting the notion that RNAi strategies may improve food safety by controlling the growth of phytopathogenic, mycotoxin-producing fungi (reviewed by Majumdar et al., 2017; Machado et al., 2018).

Despite the notable efficiency the RNAi-based technology holds for agriculture, the mechanisms underlying HIGS and SIGS technologies are inadequately understood. There is little information regarding the contribution of either plant- or fungal-silencing machinery in cross-species RNA silencing (i.e., plant and fungus) or how inhibitory RNAs translocate from the plant to the fungus after its transgenic expression or spray application. Whereas HIGS is virtually based on the plant's ability to produce mobile siRNAs (through plant Dicers [DCLs]), the mechanism of gene silencing by exogenously delivered dsRNA depends primarily on the fungal RNAi machinery, mainly fungal DCLs (Koch et al., 2016; Gaffar et al., 2019). Interestingly, recent studies revealed that AGO and DCL proteins of *Fg* contribute to fungal vegetative and generative growth, mycotoxin production, antiviral response, sensitivity to environmental RNAi, and plant disease development (Kim et al., 2015; Son et al., 2017; Yu et al., 2018; Gaffar et al., 2019). In *Fg*, two Dicer proteins (*Fg*DCL1 and *Fg*DCL2) and two AGO proteins (*Fg*AGO1 and *Fg*AGO2) were identified (Chen et al., 2015). Characterization of those RNAi core components revealed functional diversification, as *Fg*AGO1 and *Fg*DCL2 were shown to play important role in hairpin-RNA-induced gene silencing (Chen et al., 2015). In addition, we recently demonstrated that *Fg*AGO2 and *Fg*DCL1 are required for sex-specific RNAi (Gaffar et al., 2019). Moreover, *Fg*AGO2 and *Fg*DCL1 participate in the biogenesis of perithecium-specific microRNAs (Zeng et al., 2018).

Notably, we previously demonstrated that *Fg*DCL1 is required for SIGS-mediated *Fg* disease resistance (Koch et al., 2016). However, further analysis of *Fg* RNAi KO mutants revealed that all tested mutants were slightly or strongly compromised in SIGS, whereas *FgCYP51* target gene expression was completely abolished *in* Δ*dcl2* and Δ*qip1* mutants (Gaffar et al., 2019).

Together, these studies indicate a central role of RNAi pathways in regulating *Fg* development, pathogenicity, and immunity. Consistent with this notion, we assume that *Fg* RNAi components represent suitable targets for RNA spray-mediated disease control. To determine this, we generated different dsRNA constructs targeting *FgAGO* and *FgDCL* genes that were sprayed onto barley leaves. We also compared two different dsRNA design strategies; in particular, we used a tool-based prediction of suitable dsRNA construct sequences vs. a manual construct design related to current dsRNA design principles and experiences. The tool-designed dsRNA molecules, which target specific and easily accessible regions are shorter, while the manually-designed dsRNA molecules are longer and target non-overlapping regions.

MATERIALS AND METHODS

Construction of AGO1, AGO2, DCL1, and DCL2 Templates and Synthesis of dsRNA

Primers were designed to generate PCR amplicons of 658–912 bp in length for the manually-designed construct or of 173–193 bp in length for the tool-designed construct (Zhao Bioinformatics

Laboratory tool)[1], corresponding to exons of selected target genes, in which *Fg* represents *Fusarium graminearum*: *FgAGO1* (FGSG_08752), *FgAGO2* (FGSG_00348), *FgDCL1* (FGSG_09025), and *FgDCL2* (FGSG_04408) (**Supplementary Figures S1–S4**). The target gene sequences were amplified from *Fg* wt strain IFA65 cDNA using target-specific primers (**Supplementary Table S1**). The length of manually selected sequences were 658 bp for *FgAGO1*, 871 bp for *FgAGO2*, 912 bp for *FgDCL1*, and 870 bp for *FgDCL2*, while the respective tool-designed sequences were 173, 192, 182, and 193 bp in length, respectively. The respective sequences of tool- and manually-designed constructs did not overlap.

The construction of pGEMT plasmids comprised of the tool- and manually-designed target sequences was performed using restriction enzyme-cloning strategies. The first step in constructing pGEMT plasmids containing manually-designed double targets was to amplify target sequences of *AGO1*, *AGO2*, *DCL1*, and *DCL2* from the confirmed plasmids with primers containing restriction sites (**Supplementary Table S1**). The manually-designed dsRNA targeting *FgAGO1* and *FgAGO2* had a length of 1,529 bp and was therefore named ago1/ago2_1529nt. According to this scheme the other manually-designed dsRNAs were named ago1/dcl1_1570nt, ago1/dcl2_1528nt, ago2/dcl1_1783nt, ago2/dcl2_1741nt, and dcl1/dcl2_1782nt. Briefly, an *AGO2* PCR fragment was inserted between NotI and NdeI restriction sites of pGEMT plasmids containing *AGO1* or *DCL1* target sequences to generate ago1/ago2_1529nt and ago2/dcl1_1583nt constructs. The PCR fragment of *AGO1* was inserted between NotI and NdeI restriction sites of pGEMT plasmids containing the *DCL1* target sequence to construct ago1/dcl1_1570nt target plasmid. The other manually designed constructs (ago1/dcl2_1528nt, ago2/dcl2_1741nt and dcl1/dcl2_1782nt) were generated following the same procedure as described above: DCL2 PCR fragments were inserted in the AGO1 background (using NotI and NdeI), in AGO2 (using NotI and BstXI) and in DCL1 (using NotI and SalI). To construct pGEMT plasmids containing tool-designed target sequences (ago1/ago2_365nt, ago1/dcl1_355nt, ago2/dcl1_374nt, ago1/dcl2_366nt), the single targets were amplified using primers containing a restriction site (**Supplementary Table S1**), as described above. A tool-designed sequence of *DCL1* was inserted between NotI and SalI restriction sites of the pGEMT plasmid containing *AGO1* and *AGO2* targets to generate ago1/dcl1_355nt and ago2/dcl1_374nt constructs, respectively. The *DCL2* fragment was inserted between the NotI and SalI restriction sites of the pGEMT plasmid containing the *AGO1* sequence to construct ago1/dcl2_366nt. Finally, *AGO2* was inserted between the NotI and SalI restriction sites of the pGEMT plasmid containing the *AGO1* target sequence to generate an ago1/ago2_365nt construct. As a negative control a previously described dsRNA corresponding to a 720 nt long fragment of the jellyfish green fluorescent protein (GFP) was used (Koch et al., 2016).

MEGAscript Kit High Yield Transcription Kit (Ambion) was used for dsRNA synthesis by following the manufacturers' instructions using primers containing a T7 promoter sequence at the 5' end of both forward and reverse primers (**Supplementary Table S1**).

[1]http://plantgrn.noble.org/pssRNAit/

Spray Application of dsRNA on Barley Leaves

The second leaves of 2- to 3 week old barley cultivar (cv.) Golden Promise were detached and transferred to square Petri plates containing 1% water-agar. The dsRNA was diluted in 500 µl of water to a final concentration of 20 ng μl^{-1}. For the Tris-EDTA (TE) control, TE buffer was diluted in 500 µl of water, corresponding to the amount used for dilution of the dsRNA. The typical dsRNA concentration after elution was 500 ng μl^{-1}, representing a buffer concentration of 400 µM of Tris-HCL and 40 µM of EDTA in the final dilution. Leaves were sprayed using a spray flask as described earlier (Koch et al., 2016). The upper half of each plate containing ten detached leaves was evenly sprayed (3–4 puffs) with the different tool- and manually-designed dsRNAs or TE buffer and subsequently kept at room temperature. Forty-eight hours after spraying, leaves were drop-inoculated with three 20 µl drops of *Fg* suspension containing 5×10^4 conidia ml^{-1} water. After inoculation, plates were closed and incubated for 5 days at room temperature. The relative infection of the leaves was recorded as the infection area (**Supplementary Figure S5**) (by determining the size of the chlorotic lesions) relative to the total leaf area using ImageJ software (Schneider et al., 2012). We produced four biological replicates for independent sample collection. Each treatment group was compared to the TE-Buffer control using students *t*-test.

Fungal Transcript Analysis

To assess the silencing of the *FgAGO* and *FgDCL* genes, mRNA expression analysis was performed using quantitative reverse-transcription PCR (qRT-PCR). RNA extraction from the diseased leaves was performed with TRIzol (Invitrogen) following the manufacturer's instructions. Freshly extracted mRNA was used for cDNA synthesis using a qScript™ cDNA kit (Quantabio). For qRT-PCR, 10 ng of cDNA was used as a template with the reactions run in a QuantStudio 5 Real-Time PCR system (Applied Biosystems). Amplifications were performed in 7.5 µl of SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich) with 5 pmol of oligonucleotides. Each sample had three technical repetitions. Primers were used for studying expressions of *FgAGO* and *FgDCL* genes with reference to the *Elongation factor 1-alpha* (*EF1-a*) gene (FGSG_08811) and *ß-tubulin* (**Supplementary Table S1**). After an initial activation step at 95°C for 5 min, 40 cycles (95°C for 30 s, 57°C for 30 s, 72°C for 30 s) were performed. Cycle threshold (Ct) values were determined using the 7,500 Fast software supplied with the instrument. Levels of *FgAGO* and *FgDCL* transcripts were determined via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing the amount of target transcript to the amount of the reference transcripts of the *EF1-a (translation elongation-factor 1α)* and *ß-tubulin*.

siRNA Prediction

Sequences of the single manually- and tool-designed dsRNA constructs for each gene, *FgAGO1*, *FgAGO2*, *FgDCL1*, and *FgDCL2*, were split into k-mers of 21 bases and mapped to the coding sequences of the four *FgAGO* and *FgDCL* genes. The efficient siRNAs were calculated on the basis of the thermodynamic properties of the siRNA-duplex, the 5'-nucleotide of the guide strand and the target site accessibility based on the default parameters of the SI-FI software tool[2]. These parameters were: no mismatches to the target sequence, a 5'-A or -U on the potential guide strand, a higher minimum free energy (MFE) on the 5'-end of the guide strand compared to the passenger strand and good target site accessibility; the default parameters were used.

RESULTS

Spray-Induced Gene Silencing by AGO- and DCL-dsRNAs Reduces *Fg* Infection

We assessed whether *FgAGO* and *FgDCL* genes are suitable targets for SIGS-mediated plant protection strategies. Detached barley leaves were sprayed with 20 ng μl^{-1} dsRNA and drop-inoculated 48 h later with a suspension of *Fg* conidia. After 5 dpi, necrotic lesions were visible at the inoculation sites of leaves sprayed with TE buffer or non-homologous GFP-dsRNA as negative controls. All homologous dsRNAs reduced the Fg-induced symptoms, as revealed by significantly smaller lesions in detached barley leaves (**Figure 1**). Infected areas were reduced on the average by 50% compared to the control (**Figure 1**). The highest infection reduction of 60% was reached with dsRNAs targeting ago1/ago2_365nt and ago1/dcl1_1570nt (**Figure 1**). The lowest disease resistance efficiencies of 31% were shown for the ago2/dcl1_1783nt dsRNA construct (**Figure 1**).

DCL-dsRNAs Exhibited Higher Target Gene Silencing Than AGO-dsRNAs

To analyze whether the observed resistance phenotypes were provoked by target gene silencing, we measured the transcript levels of *FgAGO* and *FgDCL* genes of *Fg* grown in the infected leaf tissue by qRT-PCR. As anticipated, the relative transcript levels of targeted genes *FgAGO1*, *FgAGO2, FgDCL1,* and *FgDCL2* were reduced after the inoculation of leaves sprayed with the respective dsRNA constructs (**Figures 2A,B**), except for *FgAGO1*, if targeted with tool-designed constructs ago1/dcl1_355nt, ago1/dcl2_366nt, and ago1/ago2_365nt (**Figure 2A**). However, regarding those three constructs, we detected silencing effects for the second target gene, as the *FgDCL1* expression was reduced by 47%, *FgDCL2* by 44%, and *FgAGO2* by 52% (**Figure 2A**). The most efficient construct in terms of overall target gene silencing was ago2/dcl1_374nt, which reduced the transcripts of *FgAGO2* and *FgDCL1* by 40 and 74%, respectively, compared to the TE control (**Figures 2A,B**).

Notably, if we compared the results for the tool-designed dsRNA constructs with the manually-designed dsRNAs we observed similar results for the *FgAGO1* target-silencing

[2]http://labtools.ipk-gatersleben.de/

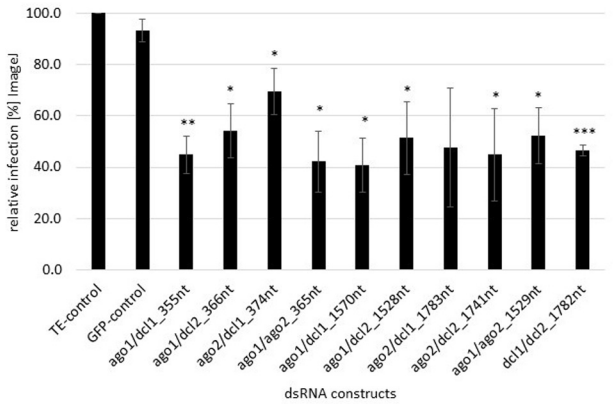

FIGURE 1 | Quantification of infection symptoms of *Fg* on barley leaves sprayed with AGO/DCL-targeting dsRNAs. Detached leaves of 3 week-old barley plants were sprayed with AGO/DCL-targeting dsRNAs or TE buffer. After 48 h, leaves were drop inoculated with 5×10^4 ml^{-1} of macroconidia and evaluated for infection symptoms at 5 dpi. Infection area, shown as the percent of the total leaf area for 10 leaves for each dsRNA and the TE control relative to the infected leaf area. Bars represent mean values ± SDs of three independent experiments. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, students *t*-test).

(**Figures 2A,B**). The constructs ago1/dcl1_1570nt and ago1/dcl2_1528nt reduced *FgAGO1* transcripts by only 17 and 29%, respectively (**Figure 2B**). Analyzing the transcript levels of *FgAGO2* revealed that: (a) the silencing efficiencies of ago2/dcl1_1783nt and ago2/dcl2_1741nt were higher than *FgAGO1* target silencing and (b) targeting both *FgAGO* genes with the ago1/ago2_1529nt construct resulted in 50% reduction for *FgAGO1* and 62% for *FgAGO*2. This, therefore, showed the highest overall *FgAGOs* gene silencing (**Figure 2B**).

Interestingly and consistent with the tool-designed target gene silencing results, we detected the strongest reduction of >70% for *FgDCL1* (**Figure 2B**). For example, ago2/dcl1_1783nt-dsRNA provoked a 79% reduction of *FgDCL1* transcripts. Target gene silencing for *FgDCL2* was also highly efficient, as use of all three constructs, ago1/dcl2_1528nt, ago2/dcl2_1741nt and dcl1/dcl2_1782nt, resulted in an ˜60% silencing efficiency (**Figure 2B**). The most efficient construct in terms of overall target gene silencing was dcl1/dcl2_1782nt, which reduced the transcripts of *FgDCL1* and *FgDCL2* by 78 and 58%, respectively, compared to control. Overall, these results suggest that silencing conferred by AGO- and DCL-dsRNAs exhibited the highest efficiency for silencing of *FgDCL1* (AVE: 70%), followed by *FgDCL2* (AVE: 58%), *FgAGO2* (AVE: 48%) and *FgAGO1* (AVE: 26%) (**Table 1**).

Manually-Designed dsRNAs Exhibit Higher Gene-Silencing Efficiencies Than Tool-Designed dsRNAs

To assess whether tool-designed dsRNA is more efficient than manually designed constructs, we directly compared target gene-silencing efficiencies of both design approaches (**Figure 3**). We observed that target gene silencing of manually-designed

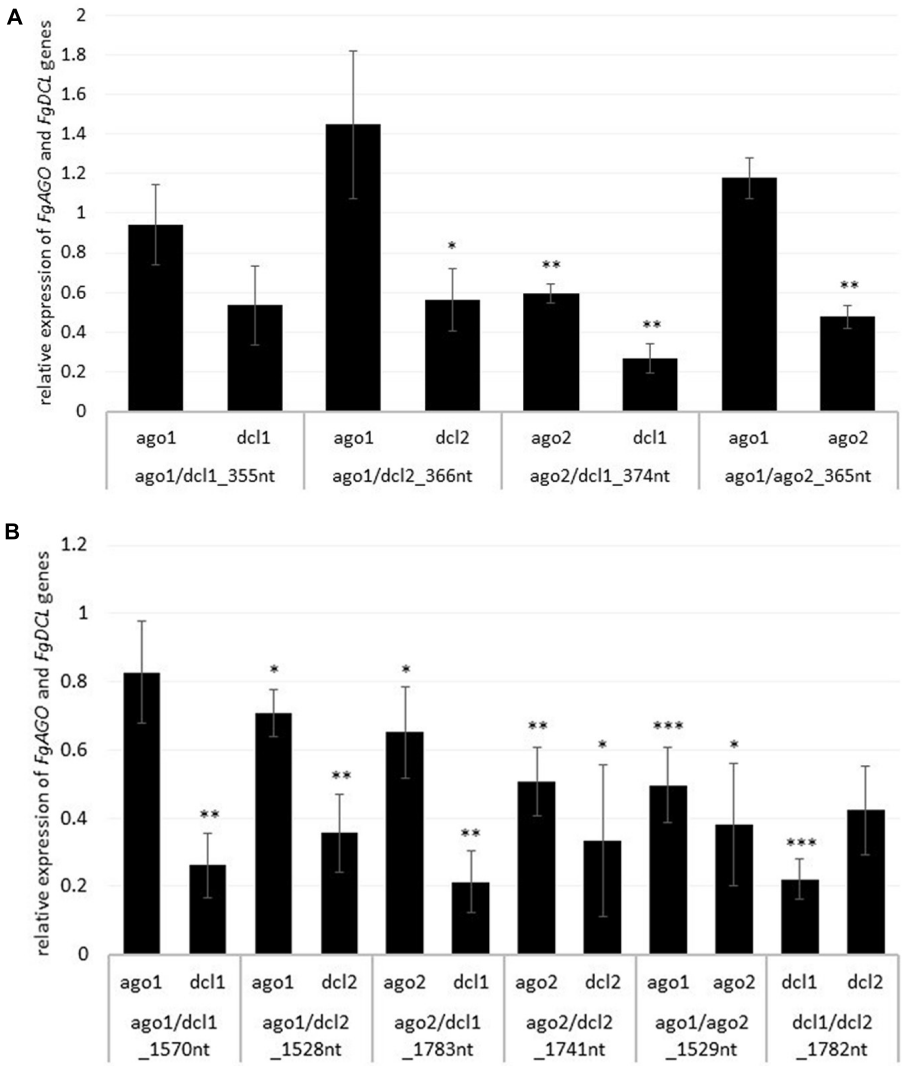

FIGURE 2 | Relative expression of the respective fungal *DCLs* and *AGOs* 5 dpi on **(A)** tool- and **(B)** manually-designed-dsRNA-sprayed leaves. The expression was measured via the $2^{-\Delta\Delta Ct}$ method in which the expression of the respective *AGOs* and *DCLs* was normalized against the fungal reference genes *EF1α (translation elongation-factor 1 α)* and *ß-tubulin*, and this Δ-Ct value was then normalized against the Δ-Ct of the GFP control. Error bars represent the SE of the four independent experiments, each using 10 leaves of 10 different plants for each transgenic line. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, students *t*-test).

constructs was superior to tool-designed dsRNA (**Figure 3**), except for *FgAGO2*, for which we found no differences between tool- or manually-designed dsRNA. Based on these findings and considering previous results, we anticipated that larger dsRNA constructs resulted in higher numbers of efficient siRNAs (Höfle et al., 2019; Koch et al., 2019). As the tool-designed constructs were <200 nt in length compared to >650 nt for the manually-designed dsRNA (**Table 2** and **Supplementary Figures S1–S4**), we calculated bioinformatically the possible siRNA hits in the *FgAGO and FgDCL* target genes for all tested dsRNA constructs (**Table 2**).

For the manually-designed dsRNA, which target different regions of the respective genes, we calculated siRNAs that were 4- to 10-fold more efficient compared to the tool-designed constructs (**Table 2**), confirming that the dsRNA precursor length probably plays a role in determining the number of derived siRNAs. For example, we predicted 49 efficient siRNAs deriving from the 912 nt manually-designed dsRNA, which targets *FgDCL1*, which is 10-fold >5 siRNA hits derived from the 182 nt tool-designed *FgDCL1*-dsRNA (**Table 2**). Notably, these differences resulted in only an overall 10% silencing efficiency decrease of the tool-designed dsRNA compared to the manually-designed constructs targeting *FgDCL1* (**Table 2**). Together, these data suggest that longer dsRNAs result in a higher number of efficient siRNAs, but there is no stringent correlation between the number of efficient siRNAs and the increase in target gene silencing (**Table 2**).

TABLE 1 | Overview of target gene-silencing efficiencies of different tested AGO- and DCL-dsRNA constructs.

		FgAGO1	*FgAGO2*	*FgDCL1*	*FgDCL2*
Tool	AGO1-DCL1	6	–	47	-
	AGO1-DCL2	No silencing	–	–	44
	AGO2-DCL1	–	41	73	–
	AGO1-AGO2	No silencing	52	-	–
	Average	6	46	60	44
Manual	AGO1-DCL1	17	–	74	–
	AGO1-DCL2	29	–	–	64
	AGO2-DCL1	–	35	79	–
	AGO2-DCL2	–	49	–	67
	AGO1-AGO2	50	62	–	–
	DCL1-DCL2	–	–	78	58
	Average	32	49	77	63

TABLE 2 | Number of efficient siRNAs and silencing efficiency of double dsRNA constructs.

Target gene	Length (nt)		Efficient siRNAs		AVE: silencing efficiency	
	Tool	Manual	Tool	Manual	Tool	Manual
FgAGO1	173	658	13	57	6	32
FgAGO2	192	871	12	58	46	49
FgDCL1	182	912	5	49	60	77
FgDCL2	193	870	9	92	44	63

These efficient sRNA are designated by the dsRNA design tool si-Fi (http://labtools.ipk-gatersleben.de).

DISCUSSION

Microbial pathogens and pests, unlike mammals, are amenable to environmental sRNAs, meaning that they can take up non-coding RNAs from the environment, and these RNAs maintain their RNAi activity (Winston et al., 2007; Whangbo and Hunter, 2008; McEwan et al., 2012). This knowledge raises the possibility that plants can be protected from pathogens/pests by exogenously supplied RNA biopesticides (for review, see Mitter et al., 2017b; Cai et al., 2018b; Dubrovina and Kiselev, 2019; Gaffar and Koch, 2019; Dalakouras et al., 2020). Possible agronomic application of environmental RNA is affirmed by the high sensitivity of *Fg* to dsRNAs and siRNAs (Koch et al., 2016). Here, we demonstrated that targeting, via SIGS, key components of the *Fg* RNAi machinery, such as *AGO* and *DCL* genes, could protect barley leaves from *Fg* infection. Our findings, together with other reports, underline that *Fg* RNAi pathways play a crucial role in regulating fungal development, growth, reproduction, mycotoxin production and pathogenicity (Kim et al., 2015; Son et al., 2017; Gaffar et al., 2019). However, the mechanistic role of *Fg* RNAi components in these processes are inadequately understood. Nevertheless, existing data suggest that there is a functional diversification of *Fg*AGO1/*Fg*DCL2- and *Fg*AGO2/*Fg*DCL1-regulated pathways (Chen et al., 2015; Son et al., 2017; Zeng et al., 2018; Gaffar et al., 2019).

Based on these findings, the dsRNAs tested in this study were designed to target *FgAGO* and *FgDCL* genes pairwise.

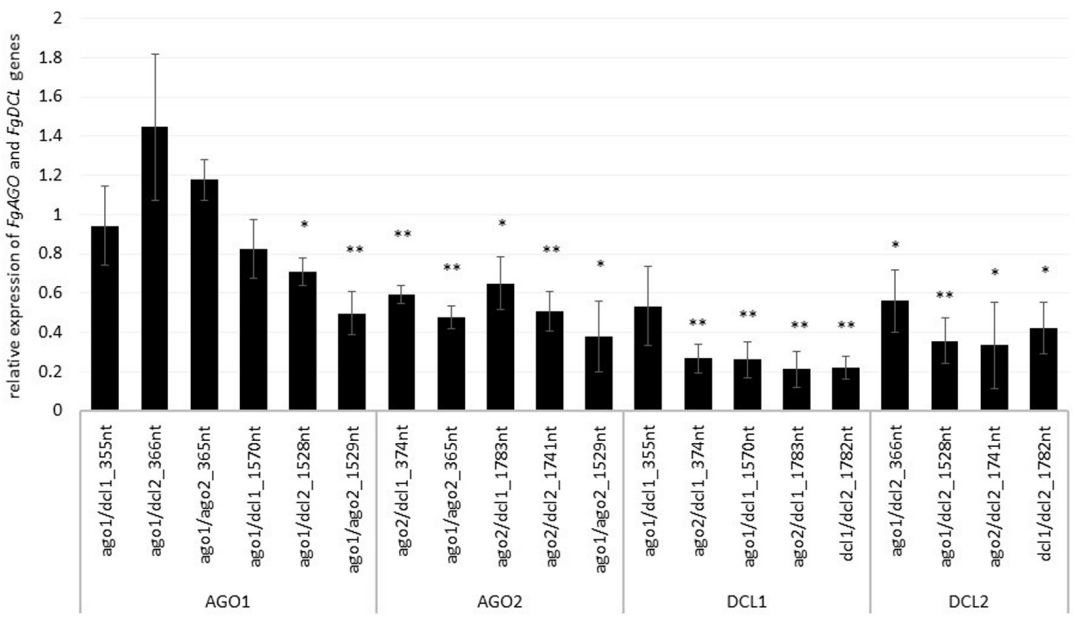

FIGURE 3 | Direct comparison of long (manual) and short (tool) constructs. Relative expression of the respective fungal *DCLs* and *AGOs* 5 dpi on dsRNA-sprayed leaves is grouped by the target gene. The expression was measured via the ΔΔ-Ct method in which the expression of the respective *AGOs* and *DCLs* was normalized against the fungal reference gene *EF1α (translation elongation-factor 1 α)* and *β*-tubulin, and this Δ-Ct value was then normalized against the Δ-Ct of the GFP control. The asterisks indicate a significant expression of the sprayed leaves in comparison to the mock-treated TE controls. Bars represent mean values ± SE of the four independent experiments.

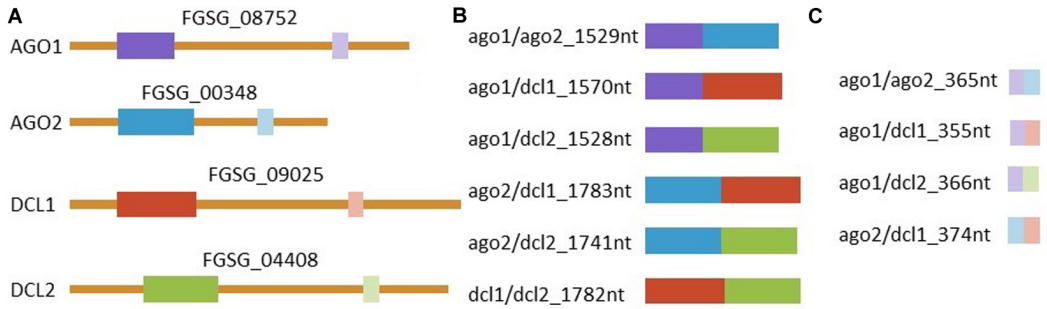

FIGURE 4 | Representation of dsRNAs and the complementary region in the corresponding genes. **(A)** Graphic representation of all four targeted mRNAs and their respective accessions with target regions marked in colors. Manually selected regions are marked in dark colors and regions selected by the pssRNAit tool[1] for better target accessibility are marked with light colors. All **(B)** manually and **(C)** tool designed dsRNAs triggers are shown. RNAs are represented correctly scaled to each other.

Thus, we generated six different dsRNA constructs covering all possible *AGO-DCL* combinations (**Figure 4**). Spraying the different dsRNAs onto barley leaves resulted in ∼50% inhibition of fungal infection for all constructs (**Figure 1**). By analyzing the silencing efficiencies of the different dsRNA constructs, we found that the expression of *FgDCLs* genes was more suppressed than *FgAGOs* genes (**Table 1**). More importantly, the expression of *FgAGO1* was completely unaffected, regardless of which dsRNA was sprayed. Based on this result, we could speculate that *Fg*AGO1 is required for binding of SIGS-associated siRNAs; thus, loss of function mediated by SIGS will not work. Of note, Δ*AGO1* mutants of *Fg* were only slightly compromised in SIGS and less sensitive to dsRNA treatments, indicating redundant functions of *Fg*AGO1 and *Fg*AGO2 in the binding of SIGS-derived siRNAs (Gaffar et al., 2019). However, further studies must explore the mechanistic role of *Fg*AGO1 in SIGS.

While our data showed that SIGS-mediated downregulation of *FgDCLs* gene expression resulted in inhibition of *Fg* infection, we cannot exclude the possibility of sprayed dsRNAs being processed by plant DCLs, which would explain the effective silencing even with silenced fungal DCLs. Consistent with this finding, previous studies demonstrated that spraying of siRNAs led to the induction of local and systemic RNAi in plants (e.g., Dalakouras et al., 2016; Koch et al., 2016). These findings are significant contributions to our mechanistic understanding of RNAi spray technology, as our previous data indicate that effective SIGS requires the processing of dsRNAs by the fungal RNAi machinery (Koch et al., 2016; Gaffar et al., 2019). Whereas HIGS mainly relies on the host plant's ability to produce mobile siRNAs (generated from transgene-derived dsRNAs), the mechanism of gene silencing by exogenously delivered dsRNA constitutes a more complex situation; for instance, the possible involvement of the silencing machinery of the host and/or pathogen (**Figure 5**). Our previous finding that unprocessed long dsRNA is absorbed from leaf tissue (Koch et al., 2016) has important implications for future disease control strategies based on dsRNA. It is very likely that the application of longer dsRNAs might be more efficient than the application of siRNAs, given their dsRNAs more efficient translocation (Koch et al., 2016). Moreover, in contrast to using only one specific siRNA, processing of long dsRNA into many different inhibitory siRNAs by the fungus may reduce the chance of pathogen resistance under field test conditions. However, RNAi-based plant protection technologies are limited by the uptake of RNAi-inducing trigger molecules, either siRNAs and/or dsRNAs; both RNA types have been shown to confer plant disease resistance independent of how they were applied/delivered (i.e., endogenously or exogenously).

Previously, we discovered that longer dsRNAs of 400–800 nt exhibited a higher gene-silencing efficiency and a stronger disease resistance than 200 nt dsRNAs (Koch et al., 2019) indicating that the quantity of siRNAs derived from a longer dsRNA precursor is simply higher. To test whether the length and/or the selected target gene sequence influences silencing efficiencies, we constructed 10 different dsRNA constructs

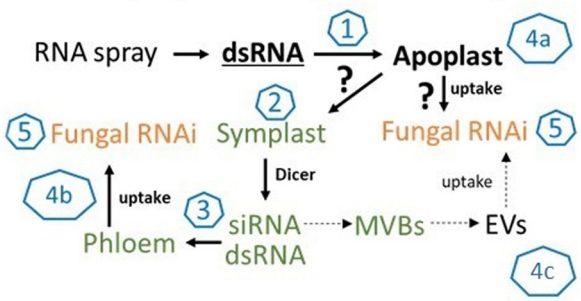

FIGURE 5 | The molecular mechanism of SIGS is controlled by the fungal silencing machinery. In summary, our findings support the model that SIGS involves: (1) uptake of sprayed dsRNA by the plant (via stomata); (2) transfer of apoplastic dsRNAs into the symplast (DCL processing into siRNAs); (3) systemic translocation of siRNA or unprocessed dsRNA via the vascular system (phloem/xylem); (4) uptake of apoplastic dsRNA (a) or symplastic dsRNA/siRNA by the fungus (b) or siRNAs from multivesicular body (MVBs) derived extracellular vesicles (EVs) (c); (5) processing into siRNA by fungal DCL.

targeting *FgAGO/FgDCL* pairs (**Figure 4**). For the design of the dsRNA constructs we used a dsRNA design tool[3] that generates dsRNAs of shorter lengths (173–197 nt), compared them to manually selected sequences (658–912 nt) and calculated the number of efficient siRNAs for each construct using si-Fi 2.1[4] *in silico* prediction tool (**Table 2**). These differences in length are inherent in the design methods and represent therefore the different design approaches. While the tool-designed RNA-trigger are designed to target a specific and well accessible region of the target mRNA the manual design approach pays little attention to these factors and is based on a more or less random selection of regions. Notably, we found that the number of efficient siRNAs derived from the longer, manually-designed dsRNAs was 4- to 5-fold higher for the constructs that target *FgAGO1* and *FgAGO2*. Moreover, the manually-designed constructs targeting *FgDCL1* and *FgDCL2* resulted in 10-fold more efficient siRNAs than the tool-designed versions (**Table 2**). However, such a correlation was only observed when we compared tool- vs. manually-designed dsRNA (<200 vs. >650 nt constructs). If we attempt to predict the number of efficient siRNAs of all the manually-designed dsRNAs, based on the length of their precursors, we obtained contrasting results. For example, the 912 nt precursor dsRNA that targets *FgDCL1* resulted in 49 efficient siRNA hits, which is approximately half of the 92 siRNA hits for the 870-nt dsRNA designed to target *FgDCL2* (**Table 2**). Importantly, the tested dsRNAs that target *FgDCL1*, which showed the lowest number of siRNAs, revealed the highest efficiencies compared to all other constructs (**Table 2**). Together, our data support the notion that longer dsRNAs tend to result in higher numbers of siRNA, although this can differ in particular cases. However, these data were obtained from *in silico* predictions; therefore, their accuracies remain unknown. Small RNA-sequencing must be performed to quantify, analyze and map the SIGS-derived siRNAs to their target genes as well as their dsRNA precursors. Besides siRNA concentration, the siRNA sequence represents a crucial determinant affecting silencing efficiency of its complementary target genes (Ossowski et al., 2008). In addition, mapping of siRNAs to their target sequence revealed processing patterns that might help to define principles for RNAi trigger design, producing effective siRNAs (Yang et al., 2013; Koch et al., 2016; Baldwin et al., 2018). Importantly, to construct our manually-designed dsRNAs, we performed a random selection of sequences complementary to the specific target genes. Moreover, to guarantee optimal silencing, we chose longer dsRNA sequences compared to the tool-designed dsRNAs. Thus, a random selection of longer target sequences, which are more effective in target silencing, tends to increase off-target effects *per se*, due to the increase in the number of different potential siRNAs (Roberts et al., 2015). Shorter target sequences, which are also specifically selected to produce potential siRNAs with a minimal potential to silence unintended targets, could greatly reduce these off-target effects. Therefore, based on our results obtained with the tool-designed dsRNAs and the work of others, we suggest using minimal-length dsRNA sequences carefully selected based on known design criteria requirements. Another possible way to achieve high silencing efficiencies while retaining high target specificity (less off-target effect) could be the construction of dsRNAs repeating a shorter tool- designed sequence several times.

Nevertheless, the number of efficient siRNAs that reach the fungus depends on the uptake efficiency of sprayed dsRNA molecules and that can differ depending on the parameters which determine the uptake efficiency, such as the stomata opening (Koch et al., 2016). Additionally, as we previously found in SIGS, the concentration of siRNAs in the target organism (i.e., fungus) can vary and mainly rely on the uptake of unprocessed dsRNA from the plant's apoplast and their processing by fungal DCLs (Koch et al., 2016; Gaffar et al., 2019). Finally, and even more important than quantities of target-specific siRNAs in determining silencing efficacy, is the target accessibility of a siRNA (Reynolds et al., 2004; Shao et al., 2007). Therefore, the design of RNAi triggers that likely mediate the efficient uptake of dsRNAs and/or siRNAs by the target pathogen is crucial in the success of SIGS as well as HIGS technologies.

Together, our results indicate that silencing fungal RNAi pathway genes, especially *DCL* genes, using SIGS efficiently increases plant disease resistance toward necrotrophic fungal pathogens, such as *Fg*. Moreover, our results support the notion that fungal RNAi-related genes in *Fg* play an essential, direct or indirect role in pathogenicity and/or virulence (Gaffar et al., 2019). These findings are consistent with other reports demonstrating that the two DCL proteins (DCL1 and DCL2) of the necrotrophic fungal pathogen *Botrytis cinerea* (*Bc*) play a central role in disease development (Wang et al., 2016). These authors showed that the application of sRNAs or dsRNAs on fruits, vegetables and flowers targeting *BcDCL1* and *BcDCL2* genes significantly inhibited gray mold disease. Of note, the same group previously discovered that *Bc* delivers sRNAs into plant cells to silence host immunity genes, a phenomenon called 'cross-kingdom RNAi (ckRNAi)' (Weiberg et al., 2013). Emerging data further suggest that some sRNA effectors can target multiple host defense genes to enhance *Bc* pathogenicity. For example, Bc-siR37 suppresses host immunity by targeting at least 15 *Arabidopsis* genes, including WRKY transcription factors, receptor-like kinases and cell wall-modifying enzymes (Wang B. et al., 2017). Moreover, one of the most destructive biotrophic pathogens of wheat *Puccinia striiformis* also delivers fungal sRNAs, such as microRNA-like RNA1 (milR1), into host cells and suppresses wheat *Pathogenesis-related 2* (*PR-2*) in the defense pathway (Wang M. et al., 2017). Notably, such ckRNAi-related sRNA effectors are produced by fungal DCL proteins, and thus SIGS of fungal DCLs abolishes sRNA production and attenuates fungal pathogenicity and growth. However, whether our findings suggest that *Fg* utilizes ckRNAi-related sRNAs to suppress host immunity needs further exploration.

More importantly, while several studies have demonstrated bidirectional ckRNAi and sRNA trafficking between plant hosts

[3]http://plantgrn.noble.org/pssRNAit/

[4]http://labtools.ipk-gatersleben.de/

and their interacting fungal pathogens (Zhang et al., 2012, 2016; Weiberg et al., 2015; Wang B. et al., 2017; Wang M. et al., 2017; Zhu et al., 2017; Cai et al., 2018a; Dubey et al., 2019; Zanini et al., 2019) the mechanisms underlying the transfer and uptake of transgene-derived artificial sRNAs (HIGS) as well as exogenously applied dsRNA (SIGS) remain elusive. Further research is needed to determine, for example: (a) how plant and fungal-silencing machinery contributes to HIGS and SIGS; (b) the nature of the inhibitory RNA that translocates from the plant to the fungus after its transgenic expression or spray application; (c) how that RNA crosses the plant-fungal interface; and (d) how dsRNA is transported at the apoplast-symplast interface. Therefore, addressing these questions is key for making RNAi-based strategies a realistic and sustainable approach in agriculture.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AK designed the study and wrote the manuscript. BW, FG, DB, and JS conducted the experiments. AK and BW analyzed all the data and drafted the figures. JS and DB conducted the RNA spray experiments. BW performed the bioinformatics analysis. All authors reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00476/full#supplementary-material

FIGURES S1–S4 | Coding sequences (CDS) of the respective *Fg* target gene with the sequences of the dsRNA marked (blue, tool-designed; red, manually designed).

FIGURE S5 | Representative pictures of barley (golden promise) leaves sprayed with 10 μg (20 ng/μl) of respective dsRNA in TE-Buffer and the control without dsRNA. DsRNA was applied on the upper half of 10 leaves and 2 days after spraying the leaves were inoculated with three 20 μl droplets of *Fg* (50,000 spores/ml). The pictures were taken 5 dpi.

TABLE S1 | Primers used in this study.

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Summary of Discussions From the 2019 OECD Conference on RNAi Based Pesticides

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RNA interference (RNAi) is a biological process in which double-stranded ribonucleic acid (dsRNA) molecules inhibit protein expression. In recent years, the application of dsRNA has been used in the development of agricultural products for pest control. The 2019 Organisation for Economic Cooperation and Development (OECD) Conference on RNAi Based Pesticides ("the Conference") brought together academic, industry, and government experts in various aspects of RNAi to discuss the current state of knowledge and topics to help in developing considerations for risk assessment. The Conference focused on environment, with some discussion of human health. Along with presentations on the use of dsRNA-based products in agriculture, government regulation, risk assessment, and a background on the Draft OECD Working Paper on "Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied dsRNA-Based Pesticides" ("OECD Working Paper"), the Conference included panel discussions from presenters at the end of each session and a larger discussion session with Conference participants on the environmental fate of dsRNA, non-target organism (NTO) risk assessment, and human health risk assessment. This paper summarizes input from presenters and Conference participants during these discussions. Key considerations from these discussions have already been incorporated into the OECD Working Paper, that once finalized and published, will facilitate regulators in evaluating externally applied dsRNA-based products for potential environmental risks.

Keywords: double stranded RNA, RNA interference, pest control, regulation, gene silencing, environmental risk assessment, non-target organisms

BACKGROUND

RNA interference (RNAi) is a biological process in which double-stranded ribonucleic acid (dsRNA) molecules inhibit protein expression, typically by triggering the enzymatic cleavage of specific messenger RNA (mRNA) molecules which are templates for protein synthesis (Fire et al., 1998; Agrawal et al., 2003). In this process, short interfering RNAs (siRNAs) derived from Dicer-mediated cleavage of long dsRNA initiate destruction of an mRNA through complementary base-pairing (Elbashir et al., 2001). This sequence-specific mode of action has been harnessed for the development of genetically modified (GM) corn for corn rootworm control (Bolognesi et al., 2012) and for targeted human and animal therapies (Vaishnaw et al., 2010). Pest control may be achieved by inducing RNAi through topical application (e.g., spraying) of dsRNA (with a nucleotide sequence developed to target a specific gene from pest or pathogen species) onto plants (San Miguel and Scott, 2015; Joga et al., 2016; Cai et al., 2018; Zotti et al., 2018). To date, no topically applied dsRNA-based pesticides have been approved for use. Guidance on regulating this technology is needed since the commercialization of these products would have implications for existing policies.

As a part of its general target of improving global economic performance, the OECD aims to establish evidence-based international standards and to find solutions to a range of social, economic and environmental challenges. OECD's Co-operative Research Programme (CRP), formed by 24 countries of the 36 OECD member nations, sponsors fellowships and conferences on biological resource management for sustainable agriculture to strengthen scientific excellence and to inform future policy decisions related in the areas of agriculture, food, fisheries and forests. To address the feasibility of siRNAs as external plant protection agents, OECD CRP organized a 2.5-day conference (held on 10–12 April 2019 in Paris, France), and summarized the current state of knowledge and ongoing developments that are relevant for the regulation of dsRNA-based pesticides. There were 57 participants from academia (∼22%), industry (∼21%), and government (∼57%). Invited speakers included experts in various aspects of RNAi, and their presentations summarized product developments, environmental fate, exposure to externally applied dsRNA in NTOs, lessons from human therapeutic use of dsRNA, and key points from previous regulatory reviews of dsRNA-based crop traits.

DISCUSSION

The summaries captured here represent the varied input from multiple participants during the Conference discussion sessions and do not necessarily reflect consensus views. In the interest of space, comments from participants have been consolidated and edited for length and clarity. Content in the text is attributed using the sector of the speaker affiliation in parentheses.

Session 1: Summary of the State of the Art: dsRNA Product Use in Agriculture

A summary of the scientific background of dsRNA products in agriculture was provided by scientists from academia, industry, and government.

The use of bioinformatics analyses for predicting off-target effects and unintended gene silencing in NTOs was the primary focus of the group discussion. The participants noted that sequence homology between a dsRNA and a mammalian transcript does not necessarily lead to a silencing effect. In mammals, there is a high rate of potential matches of a dsRNA with a target gene for any dsRNA of ∼300 base pairs (bp) or more. This is generally due to the low target site specificity in mammals, where a short (7–8 bp) 'seed' region of an siRNA is sufficient for binding to transcripts and subsequent silencing. The disconnect between predicted dsRNA/transcript sequence homology and physiological effects in part is due to barriers that prevent dsRNA uptake by mammalian cells and the relatively low abundance of discrete siRNAs derived from a single dsRNA. The participants acknowledged that multiple mechanisms underlie the reported disconnect and noted differences between organisms that share a common microRNA/siRNA pathway (i.e., mammals) and those with different pathways (i.e., insects).

The concentration of individual siRNAs is another important parameter for predicting off-target effects. The risk to NTOs may be minimal if individual siRNAs are below the femtomolar range. Off-target effects may be limited by the use of low concentrations of a dsRNA preparation yielding correspondingly low concentrations of each unique individual siRNA, in contrast to a large amount of a single siRNA. Another advantage of longer dsRNAs (>60 bp) is the ability to design a molecule which can target several transcripts at a time.

A comparison of plant-produced dsRNA to spray applications of dsRNA for pest control was discussed. Generally, the dsRNA produced in genetically modified plants (GMPs) is processed within the plant into siRNAs, which are subsequently 3′ end-methylated and therefore more stable. The GMP strategy could result in less contact with NTOs and less potential for drift, runoff, or movement of the dsRNA to other environmental compartments compared to spray applications. In contrast, the potential for environmental spread via transgene escape to non-GM counterparts is not evident for spray applied dsRNAs. However, multiple factors (e.g., specificity, route of exposure, responsiveness to environmental RNA) were considered more relevant for risk assessment than a very general comparison of the method of deployment of RNAi in agriculture.

In this context, the question of when to quantify the dsRNA for exposure measurement arose, especially given that dsRNA-based pesticides may take longer to display efficacy than conventional pesticides. The group stressed that a time profile for dsRNA exposure is needed. For spray applications, there was consensus that formulation is an important factor to be addressed in the OECD Working Paper because it is essential to the stability and uptake of dsRNA.

Session 2: Summary of Regulatory and Risk Assessment Experience With dsRNA-Based Products

While research on the mechanisms of action of dsRNA is important, the group consensus was that the importance for risk assessment lies in identifying potential hazards rather than the mechanisms underlying those hazards. Additional emphasis should be placed on the selection of NTOs to conduct toxicity testing for ecological risk assessments, paying close attention to the possibilities for environmental and systemic RNAi in test species to avoid false negative results.

The role of bioinformatics in assessing potential hazards was a recurring theme. The use of bioinformatics is believed to have limited value in assessing off-target effects due to the variability in environmental exposure across organisms, barriers to systemic exposure, and differences in RNAi machinery between organisms. There was group consensus that sequence information alone should not and cannot be used as the sole predictor of effects on NTOs. However, while bioinformatics is of some limited value for hazard evaluation, it is critical in the design phase of dsRNA products and for selecting NTOs to study.

Protocols for addressing hazards with dsRNA-based products require revision compared to those for conventional pesticides because dsRNA-based products may take longer to display efficacy. Any evaluation needs to account for this time lag by extending the study period. Additionally, any evaluation of a dsRNA-based pesticide should address the degradation of the dsRNA over time. Effective use of a dsRNA-based pesticide may require a recharging application as degradation is known to occur.

Issues of public perception of dsRNA were raised. dsRNA-based pesticides are molecular biological products, but not considered as genetic modification technology since the nucleotide sequences in dsRNA-based pesticides do not code for protein and are not inserted into the genome and are not heritable like transgenes (Academia). (Authors note. Therefore, they do not fall under the scope of various gene technology regulations in certain OECD countries regardless of their slightly different definitions of GM organisms.) However, there is concern that the public may misunderstand RNAi-based pesticides as a "new genomic technique" and this may create an obstacle to public acceptance (Academia). Participants discussed this topic from a variety of perspectives (**Table 1**, Question 1).

Session 3: Discussion Themes

Environmental Fate

The potential for formulation to increase persistence of a dsRNA product requires special consideration regarding its environmental fate. The European Union (EU) has protocols to address products that persist, and these protocols could be relied on if a formulation increases dsRNA persistence. The need to reapply a dsRNA product with low environmental persistence may increase exposure to organisms that typically require repeated contact with a pesticide.

The group discussed the need to clarify whether the active ingredient or the formulation is tested both in pre- and post-market assessments. A focus on biologically based analytical methods rather than studies designed solely for understanding the chemistry was proposed.

In general, environmental fate and exposure considerations for various types of use patterns (e.g., foliar sprays, seed treatment) are the same for dsRNA as for conventional pesticides. Industry members suggested that a product or active ingredient should not be assessed differently than another similar product just because they have a different formulation (Industry). Additional industry input suggested that the formulated product should always be used in field trials. Regulators from the United States Environmental Protection Agency (US EPA) and the EU both expressed concern about exposure routes and how testing requirements may change with different formulations (**Table 2**). Application of a hazard paradigm for protected species and the ecosystem as well as the use of the endpoints already established for the risk assessment of conventional pesticides was discussed.

Concern about how the RNA sequence may change degradation rates was raised (Industry). However, in the case of a naked dsRNA, the degradation kinetics should be similar regardless of the sequence (Industry). Industry members were asked what protocols they plan to use to assess persistence. Bayer plans to look at degradation in the formulated versions of their products (Industry). The use of established laboratory protocols for studying the persistence of *Bacillus thuringiensis* (Bt) proteins was proposed (Industry).

Both industry and academic participants emphasized that a relatively large body of literature exists about the mechanism of action for dsRNA relative to conventional pesticides. Concerns about the impact of additional data requirements on creating a regulatory burden for industry were raised by industry and academic partners (Industry; Academia). A discussion ensued on the potential for extrapolating environmental data obtained with one dsRNA product to others, and how much information is needed to reach a satisfactory understanding of environmental fate. A definitive conclusion was not reached.

Non-Target Organisms (NTOs)

The potential for exposure and responsiveness to environmental RNAi were seen as the first parameters to consider in the risk assessment of external dsRNA applications before looking at sequence data.

The potential uptake of dsRNA by mammals after oral exposure is likely to be low due to substantial barriers in the oral and dermal uptake pathways. Research on barriers to invertebrate oral and topical uptake is limited. Barriers to uptake identified in mammals are assumed to apply across vertebrate species because the molecular mechanisms show conservation from fish to mammals (Academia); similarly, the literature shows a high degree of conservation in the vertebrate digestive system (Industry). Two regulatory studies exposed mice and rats to high doses of dsRNA and no effects were observed (Industry). However, pharmaceutical data, often derived from studies using mice, are based on peritoneal administrations (i.e., injection)

TABLE 1 | Responsive discussions to questions raised by participants.

1. How can governments, regulators, and industry convey ideas about this new technology to the public? What is the best way to market RNAi technology so that it is not incorrectly perceived as genetic modification? How can this clear distinction be communicated openly?
• Researchers, regulators, and industry need to be transparent and accessible in communications, especially when mentioning "DNA", "RNA", or "genes" (Academia). • There was consensus about the need to initiate conversations with the public early. Academic scientists highlighted the need to engage in dialogue with social policy, regulatory agencies, and social scientists. • Participants discussed this issue at some length, recognising that, regardless of scientific consensus on safety, balancing the need for transparency with communicating scientific uncertainty and risk to the public is a challenge. • In Europe, the definition of genetic modification specifies living organisms. If dsRNA is purified, it cannot be legally defined as genetically modified (Government Regulator). However, the public may not see this distinction (Government Regulator). • An industry scientist pointed out that part of the issue with public acceptance of genetically modified plants is that the field is perceived to be dominated by large companies (Industry). This may be less of an issue with RNAi-based products because of the cost for producing this technology is substantially lower and broad interest from diverse developers exists (Industry).
2. Are there safety concerns with nanoclay particles?
• A range of nanomaterials and their safety profiles have been investigated (Academia). An example of a nanoclay is layered double hydroxide (LDH), which has characterized nanometrology properties and is biocompatible and biodegradable (Academia). • Plant cell walls have size exclusion limits. Because the LDH component of BioClay (a complex of LDH and dsRNA) ranges from 20 – 80 nm, which exceeds the pore size on plant cell walls (< 5 nm), it is likely excluded from entering plant cells and may just act as a carrier that slowly releases dsRNA on the plant surface (Academia).
3. Is there evidence of dsRNA entering a plant's RNAi system or down-regulating an endogenous gene?
• A number of papers report dsRNA-generated systemic resistance to viruses and fungi without mechanical inoculation at the point of dsRNA application, implying that dsRNA can enter the plant through an intact surface, possibly via the stomata. The nature of this process is undetermined. • As topically-applied dsRNA has repeatedly generated successful systemic virus resistance, this suggests that dsRNA enters the plant and is taken up by the RNAi system. • Participants were not aware of any publications that demonstrate dsRNA spray-induced reduction of endogenous gene expression. The mechanism for long distance transport of topical dsRNA-derived silencing signals is undetermined.

TABLE 2 | Comparison of comments on testing for environmental assessments from various governmental or multi-governmental bodies.

European Union (Two step approach)		**United States Environmental Protection Agency**
First step: The European Food Safety Authority (EFSA) assesses an active substance including one representative formulation that the applicant proposes. The active substance will be authorized by the EU Commission.	Second step: Member State assesses each final plant protection product whose active ingredient has been authorized in the first step. The basic consideration is that differences between product formulations can alter its environmental behavior and ecotoxicological potential.	The US EPA uses a case-by-case approach and a tiered risk assessment similar to their method for evaluating biochemical pesticides.

that are less relevant to the exposure pathways of dsRNA-based pesticides (i.e., ingestion and inhalation) (Industry). For insects, a point was raised about the lack of "negative data" in the published literature (i.e., when exposure to dsRNA did not lead to a biologically meaningful effect) (Academia). While it may be possible to generalize the applicability of barriers to dsRNA uptake identified in mammals to other vertebrates, it is currently not possible to predict responsiveness across invertebrate taxa to environmental dsRNA. The group stressed that siRNAs are not taken up by most insects and their presence in the environment generally has no biological effect (author note – short dsRNAs < 30 base-pairs have, however, been effective in some feeding assays.). However, it was noted that arthropods display variable responsiveness to long dsRNA and it is difficult to generalize susceptibility to RNAi, even among closely related arthropod species.

Before doing functional testing for investigating potential agricultural uses of dsRNA, the cellular components of RNAi in the pest or NTO of interest should be examined. Evolutionary development appears to be monophyletic for silencing and amplification of the RNA signal, and Dicer enzymes among vertebrates are fully conserved.

Bioinformatics methods are likely to be useful in the design phase of species-specific dsRNA molecules and in the identification of NTOs for testing (Industry). Several group members expressed the value of examining off-target genome and transcriptome data for regions of homology to the dsRNA, although full genomes of many target species and NTOs have not been sequenced. For instance, evaluation of *DvSnf7* dsRNA-expressing corn used two approaches to bioinformatics but was limited by the number of species that could be tested (Academia).

The length of nucleotides needed in bioinformatics alignments was discussed and a minimum contiguous match between a dsRNA and an off-target transcript of ∼19 nt was proposed, based on the scientific literature (Academia). If there are no contiguous nucleotide matches of ≥19 nt to an off-target sequence, it can be assumed that there will be no sequence-dependent effect of the dsRNA on the NTO (Academia). For a highly specific construct, it was proposed that closely

related species should be tested (Academia). If a closely related species is not impacted, it is less likely a more distant one would be. The discussion on bioinformatics largely reiterates that which will be contained within the OECD Working Paper.

A challenge with using bioinformatics data is choosing the NTO of interest (Academia). Considering the regulatory process, ecological data should be used to determine which species actually live in the environment that contains the target pest (Academia). Additionally, testing organisms at various life stages may be necessary due to differential sensitivities to RNAi depending on the life stage (Government Regulator). Testing organisms from the environment of interest may not be necessary because test guidelines already specify the use of surrogate species; furthermore, testing animals from the actual environment of interest could raise concerns about biodiversity loss (Industry).

Human Health Risk Assessment (HHRA)

Concerns about the potential exposure of sensitive populations (i.e., children, the elderly, and the immune-compromised) to dsRNA-based pesticides were explored. No significant issues were raised regarding the safety of the technology to the health of sensitive populations. An important consideration discussed was the potential exposure of applicators and bystanders to dsRNA-based pesticides. It was noted that published studies relevant to the potential exposure of applicators and bystanders used doses of dsRNA that are substantially higher than expected exposure levels after field application in agriculture (expected to be approximately 0.5–1.0 gram dsRNA per hectare). Additionally, long dsRNAs are considered to have less potential for sequence-dependent off-target effects than siRNA. The impact of RNAi-based pesticide formulations on exposure was also discussed.

Unlike siRNA, long dsRNA has the potential to provoke an interferon response, which can be independent of the sequence. Because of this, bioinformatics is unlikely to be a useful method for informing the HHRA. It was noted that, because of the potential for an immune response, clinical studies so far have focused on shorter RNAs designed to bypass this response. Clinical data using long dsRNA may be lacking. Sequence-independent immune responses, if they occurred at all, were speculated to produce symptoms similar to mild influenza.

Evidence from human clinical studies suggests that systemic exposure of mammals to dsRNA when dsRNA is applied in the field as a pesticide may be quite low. Unfortunately, research yielding no adverse effects is not commonly published; the few available published animal studies on dsRNA include an acute and subchronic toxicity study and three repeat-dose toxicity studies. None of the studies demonstrated any adverse effects (including for sensitization and irritation) when dsRNA was administered orally. Additionally, any adverse impacts that have been observed from dsRNA were temporary and did not permanently impact the immune system.

The use of formulations to enhance cellular uptake may call for additional considerations in the HHRA, since it is likely that product formulations will increase the persistence or systemic uptake of a dsRNA active ingredient. For example, when nanomaterials are present in formulations for the purpose of enhancing cellular uptake of dsRNA, increased dermal and/or inhalation toxicity may occur. However, not all nanomaterials enhance the cellular uptake of dsRNA (**Table 1**, Question 2). A case-by-case consideration of the intended product's formulation will help determine whether additional data are required.

Exogenously applied dsRNA pesticides are likely to be applied using the same methods as "conventional" pesticides. Consequently, the same exposure routes are probable. Populations most likely to be exposed are farm workers applying the product and bystanders in the target area during or following product application. The primary exposure pathways for workers and bystanders are dermal contact and/or inhalation. In the absence of a regulatory policy relating to personal protective equipment for dsRNA-based pesticides, requirements will be determined on a case-by-case basis.

For the general population, dietary consumption of treated plants and/or derived commodities will likely be the dominant exposure pathway. Regarding potential human health impacts, it is important to note that dsRNA applied to plants for pest control is not integrated into the plant genome or amplified. Rather, depending on the delivery method and on the targeted pest or pathogen dsRNA may be taken up by the plant cell and processed. Unprocessed dsRNA as well as processed siRNAs are delivered to targeted pests or pathogens to trigger the RNAi pathway within the target pest. The fate of any remaining dsRNA and whether it accumulates in plants is currently unknown (**Table 1**, Question 3). Even if dsRNA residue persists in the plant, there are significant physiological and biochemical barriers limiting systemic exposure to dsRNA following oral ingestion. Studies from human health research suggest that some conjugation, encapsulation or chemical modification of dsRNA is necessary to facilitate its trans-membrane movement and to reduce its otherwise rapid renal clearance. However, the potential accumulation of dsRNA in plants may warrant consideration for dietary intake following multiple applications particularly close to harvest, or when persistent formulations are applied.

An additional Working Paper addressing HHRA of dsRNA was proposed to address the development of dsRNA-based human therapeutics and the potential for human health risks based on the outcomes of these studies.

CONCLUSION

The considerations arising from the Conference discussions were varied and represented multiple perspectives. The potential for exposure of NTOs as well as responsiveness to environmental RNAi were seen as the first parameters to consider in the

assessment of external dsRNA applications. While sequence information is useful in the design phase and in the selection of NTOs for testing, bioinformatics cannot be used as a stand-alone predictor of off-target effects. Protocols for addressing hazard with dsRNA-based products require some revisions compared to how they are carried out for conventional pesticides (e.g., by extending the study period) since dsRNA-based products may take longer to display efficacy. Evaluations of dsRNA-based pesticides should include monitoring for degradation over time. The impact of product formulation on environmental persistence of dsRNA and uptake by non-target organisms requires consideration. While it may be possible to generalize the applicability of barriers to dsRNA uptake identified in mammals to other vertebrates, it is currently not possible to predict responsiveness across invertebrate taxa to environmental dsRNA. Considerations arising from the Conference will be incorporated into the OECD Working Paper(s) as appropriate.

AUTHOR CONTRIBUTIONS

EH, AD-P, SM-C, and SF were rapporteurs at the Conference. MS, MM, and SM-C consolidated the proceedings. All authors critically revised the manuscript and contributed to the writing. All authors contributed to manuscript revision, read and approved the submitted version.

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Assessing the Risks of Topically Applied dsRNA-Based Products to Non-target Arthropods

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RNA interference (RNAi) is a powerful technology that offers new opportunities for pest control through silencing of genes that are essential for the survival of arthropod pests. The approach relies on sequence-specificity of applied double-stranded (ds) RNA that can be designed to have a very narrow spectrum of both the target gene product (RNA) as well as the target organism, and thus allowing highly targeted pest control. Successful RNAi has been reported from a number of arthropod species belonging to various orders. Pest control may be achieved by applying dsRNA as foliar sprays. One of the main concerns related to the use of dsRNA is adverse environmental effects particularly on valued non-target species. Arthropods form an important part of the biodiversity in agricultural landscapes and contribute important ecosystem services. Consequently, environmental risk assessment (ERA) for potential impacts that plant protection products may have on valued non-target arthropods is legally required prior to their placement on the market. We describe how problem formulation can be used to set the context and to develop plausible pathways on how the application of dsRNA-based products could harm valued non-target arthropod species, such as those contributing to biological pest control. The current knowledge regarding the exposure to and the hazard posed by dsRNA in spray products for non-target arthropods is reviewed and suggestions are provided on how to select the most suitable test species and to conduct laboratory-based toxicity studies that provide robust, reliable and interpretable results to support the ERA.

Keywords: ecosystem services, environmental risk assessment, hazard, exposure, pathways to harm, problem formulation, species selection, tiered risk assessment

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INTRODUCTION

RNA interference (RNAi) is a mechanism of gene silencing present in most eukaryote organism to regulate gene expression (Hannon, 2002). The silencing effect can be triggered by double-stranded RNA (dsRNA), is RNA sequence-specific, and makes use of the core RNAi machinery to degrade complementary RNA molecules. RNAi thus provides a tool that can be designed to affect and control insect pests in a highly specific manner by targeting genes that are essential for the survival of the species (Xue et al., 2012; Burand and Hunter, 2013; Zhang et al., 2017; Liu et al., 2020). In an agricultural context the technology may also be deployed to increase the sensitivity of pests or

vectors to chemical insecticides (e.g., Killiny et al., 2014; Bona et al., 2016) or to protect beneficial species from viral diseases (Vogel et al., 2019).

For application as a pest control tool, the active dsRNA molecule has to enter and affect the target pest. This can be achieved by two main ways of application. First, dsRNA can be produced *in planta*, which requires genetic engineering (GE) of the plant. The first product of that kind has recently been approved by US regulators in June 2017[1]. This particular GE maize event (MON87411) produces a dsRNA targeting the Snf7 protein in the Western Corn Rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae), which is crucial for the transport of transmembrane proteins. Suppression of the *Snf7* gene leads to increased larval mortality and consequently to reduced root damage (Bolognesi et al., 2012). The RNAi trait is combined with the Cry3Bb1 protein for improved target pest control and resistance management (Levine et al., 2015; Head et al., 2017). Second, the dsRNA molecules can be applied externally, for example in irrigation water or through trunk injections (Hunter et al., 2012; Li et al., 2015a; Niu et al., 2018; Kunte et al., 2020), in food-baits (Zhou et al., 2008; Zhang et al., 2010), by using delivery systems such as micro-organisms, viruses, nanocarriers (Kunte et al., 2020; Vogel et al., 2019), or topically as spray applications (San Miguel and Scott, 2016).

Two major challenges have been identified for implementing the RNAi-based technology in pest control. First, the target organisms have to ingest intact and biologically active dsRNA molecules in order to trigger an RNAi response. While RNAi has been observed in a number of insect species belonging to various orders, the effectiveness of dietary RNAi (derived from ingested dsRNA) is less clear (Baum and Roberts, 2014). Second, there is evidence that resistance is not developed against a specific dsRNA molecule but to components in the dsRNA uptake machinery in the intestinal tract or in the dsRNA processing machinery. For example, Khajuria et al. (2018) demonstrated for *D. v. virgifera*, that resistance to dsRNA targeting *Snf7*, was due to the fact that cellular uptake was prevented.

Despite those challenges, effective dsRNA-based spray products that cause specific toxic effects on selected arthropod pest species are expected within the next few years (Hogervorst et al., 2018; Taning et al., 2020) and our perspective will focus on this method of application.

ENVIRONMENTAL RISK ASSESSMENT

As pesticides, dsRNA-based sprays are regulated stressors that have to pass an environmental risk assessment (ERA) before being commercially released to ensure that their use causes no unacceptable harm to the environment. Given the novel mode of action, the regulatory and data requirements are discussed internationally (Auer and Frederick, 2009; US EPA, 2014; Roberts et al., 2015).

Early in the ERA, in a step called "Problem Formulation," the protection goals set by environmental policy need to be identified, and operational protection goals and plausible pathways on how the stressor of concern could harm those protection goals (i.e., pathways to harm) are defined (Raybould, 2006; Gray, 2012; Craig et al., 2017; Raybould et al., 2019). Based on these "Pathways to Harm," testable risk hypotheses can be derived, existing relevant information is collected and required data are identified. The aim of this process is to ensure that any decision taken is made in a traceable and transparent manner. While experience has been gained with applying problem formulation to the ERA of GE plants, the concept is equally applicable to other stressors, including dsRNA-based pesticides (Devos et al., 2019; Raybould and Burns, 2020).

For plant protection products such as dsRNA-based sprays, "biodiversity" is an important environmental protection goal, which is found in policies of most jurisdictions. However, this term is very general and thus specific (operational) protection goals need to be defined that can then be addressed in the scientific risk assessment. Such operational protection goals delineate the components of the environment that are valued and should be protected, including details on the location, the exact time period, and the maximum tolerable impact (Nienstedt et al., 2012; Sanvido et al., 2012; Devos et al., 2015). In this respect, it has been proposed to categorize biodiversity in categories of valued ecosystem services ("ecosystem service concept") as defined for example in the Millennium Ecosystem assessment (Millennium Ecosystem Assessment [MEA], 2005; Gilioli et al., 2014; Devos et al., 2015; European Food Safety Authority Scientific Committee, 2016; Maltby et al., 2017a,b). In the case of arthropods this includes regulating services (e.g., biological pest control, pollination), cultural services (e.g., protected species), and supporting services (e.g., arthropods that contribute to nutrient cycling).

Once the components of the environment to be protected are identified, plausible pathways to harm can be constructed. In **Figure 1** such pathways to harm are defined for the protection goal "biological pest control" that is provided by predators and parasitoids, which may be affected by the application of a dsRNA-based spray. For a spray product to cause harm to the protection goal, a line of events or steps has to occur. If one can conclude with high certainty that one or more of the steps are unlikely to happen, the pathway is interrupted, which allows to conclude that the risk to biological control is negligible (Raybould et al., 2019). Thus the different steps can be tested or assessed in the ERA to characterize the risk. In principle the steps either relate to exposure, the likelihood that non-target species actually ingest sufficient amounts of biological active dsRNA, or hazard, which relates to the sensitivity of the non-target species to dietary RNAi. These two aspects of the risk equation will be discussed in the following sections.

EXPOSURE OF NON-TARGET ARTHROPODS TO dsRNA IN SPRAY PRODUCTS

Non-target arthropod species could directly be exposed to dsRNA in spray products when consuming treated plant material in

[1]https://www.epa.gov/pesticide-registration/epa-registers-innovative-tool-control-corn-rootworm

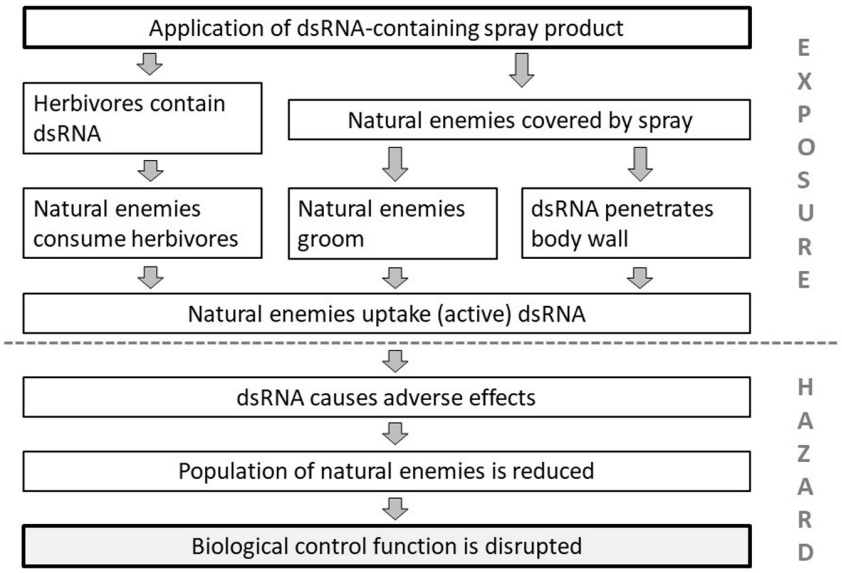

FIGURE 1 | Plausible pathways to harm. Steps on how the application of a dsRNA-based spray insecticide could cause harm to the protection goal of "biological pest control" by affecting arthropod natural enemies (predators and parasitoids).

the field or outside the field in case of spray-drift, through contact with soil and water or topical application and indirect when feeding on arthropods that have been exposed. While the plant cuticle and also the cell walls limit the uptake of spray-applied dsRNA into the plants, there is some evidence for uptake and transport in the vascular system of bioactive dsRNA (Koch et al., 2016), which can be further enhanced by high pressure spraying (Dalakouras et al., 2016) or particular carriers (Mitter et al., 2017).

In general, stability of naked dsRNA in the environment is very low. Degradation of dsRNA within 2 days has been reported for soil and aquatic environments (Dubelman et al., 2014; Fischer et al., 2016, 2017; Bachman et al., 2020) although partial adsorption to soil particles will also play a role (Parker et al., 2019). Degradation appears neither to be affected by dose (Dubelman et al., 2014) nor by length or structure of the dsRNA molecule (Fischer et al., 2016). There is some indication that degradation of dsRNA molecules is reduced on plant surfaces (Tenllado et al., 2004; San Miguel and Scott, 2016). The persistence of dsRNA in formulated spray products is difficult to predict since the active ingredient is likely to be stabilized to prevent abiotic and biotic degradation. For example, Mitter et al. (2017) recently demonstrated that pathogen-specific dsRNA targeting plant viruses could be detected for more than 30 days after application when loaded on layered double hydroxide clay nanosheets. Thus, the formulation in which the molecule is applied has to be considered in the exposure assessment (Bachman et al., 2020).

The routes and duration of non-target organism exposure to dsRNA in sprayed products will depend on a number of factors, including: (1) application rate of the active ingredient, (2) application timing, (3) application method, (4) number of applications, (5) off-site movement of applied dsRNA, and (6) stability and persistence of exogenously applied dsRNA following application (US EPA, 2014).

For predators and parasitoids we have identified three main routes of exposure (**Figure 1**). The first, and the most likely route is indirect, through their prey or hosts. Herbivores can be covered by the spray or ingest the dsRNA when feeding on the treated plants. It remains to be confirmed, however, that dsRNA ingested by a herbivore is still biologically active when passed on to the next trophic level. To our knowledge, cross-species transfer of biologically active dsRNA has only been reported in one study, i.e., between honey bees (*Apis mellifera*, Hymenoptera: Apidae) and parasitic mites, *Varroa destructor* (Acari: Varroidae) (Garbian et al., 2012). The second potential route of exposure of natural enemies is through the insects' integument. There is some evidence that dsRNA applied topically can penetrate the insect's body wall, i.e., via the inter-segmental membranes, and cause an RNAi response. The first case of this nature was reported for *Aedes aegypti* (Diptera: Culicidae) by Pridgeon et al. (2008). Penetration has also been demonstrated for larvae of *Ostrinia furnacalis* (Lepidoptera: Crambidae) using fluorescent dsRNA albeit at very high concentrations of 0.5 µl of 0.5 µg/µl fluorescent labeled dsRNA per larva (Wang et al., 2011). However, it is difficult in such topical application studies to rule out that the dsRNA molecules entered the body through the spiracles rather than through the integument. However, there is evidence that the penetration efficiency can be enhanced by altering the formulation in which the dsRNA is applied. For example, in the case of the soybean aphid *Aphis glycines* (Hemiptera: Aphididae) penetration efficiency was significantly enhanced using a nanocarrier in combination with an amphiphilic periphery detergent to increase the attachment of the droplets to the insect cuticula (Zheng et al., 2019). In a recent study, Niu et al. (2019) observed the uptake of dsRNA topically

applied to *Acyrthosiphon pisum* (Hemiptera: Aphididae) within 12 min. As a third route of exposure, insects might also ingest the molecule during grooming after they have been covered by dsRNA after a spray application. While some predators also feed on green plant tissue when prey is scarce (Lundgren, 2009) we regard this route of exposure as negligible.

Dietary uptake of dsRNA, does not necessarily mean that the molecule is still biologically active. Extraoral digestion is know from many predatory arthropods including spiders, lacewing larvae and predatory bugs (Cohen, 1998; Zhu et al., 2016; Walter et al., 2017). According to Cohen (1995) at least 79% of predaceous land-dwelling arthropods use extra-oral digestion. For example, it has been demonstrated for the plant bug *Lygus lineolaris* (Hemiptera: Miridae) that dsRNA molecules are completely digested to monomers by endonucleases in the saliva prior to ingestion (Allen and Walker, 2012).

HAZARD POSED BY dsRNA

In principle, ingested dsRNA can pose a hazard to a non-target arthropod in two ways, i.e., sequence-specific and sequence-unspecific. Mechanisms that have been suggested as a cause of sequence-unspecific effects of ingested dsRNA are first, the induction of a general immune response since RNAi is a component of the innate antiviral immunity response and second, a saturation of the RNAi machinery, i.e., the dsRNA processing enzymes (Dillin, 2003; Christiaens et al., 2018a). While saturation of the RNAi machinery has been observed in animals (mice and cell cultures) at high doses (US EPA, 2014), it has not yet been reported in arthropods (Miller et al., 2012; Christiaens et al., 2018a). DsRNA-triggered general immune responses, e.g., the upregulation of dsRNAase, have been observed in honey bees (*Apis mellifera*, Hymenoptera: Apidae) (Flenniken and Andino, 2013; Brutscher et al., 2017), bumble bees (*Bombus terrestris*, Hymenoptera: Apidae) (Piot et al., 2015), and the silkworm (Liu et al., 2013). There is evidence from feeding studies that high doses of dsRNA can boost a sequence-unspecific response in ladybird beetles (Coleoptera: Coccinellidae) (Haller et al., 2019). But comparable doses (of the same construct) did not cause such effects in other arthropod species studied (Pan et al., 2016; Vélez et al., 2016). Sequence-unspecific effects have also been observed for dsGFP in honey bees, *A. mellifera*, in feeding and injection studies (Jarosch and Moritz, 2012; Nunes et al., 2013). In summary, while there is no evidence that dsRNA can cause a saturation of the RNAi machinery in arthropods, high doses of dsRNA may affect the fitness of non-target arthropod species in a sequence-unspecific way through a stimulation of the immune system. Consequently, from an ERA perspective, non- and off-target effects of the dsRNA that are sequence specific are of much more concern and will be the focus of the following description.

After ingestion of dsRNA molecules, a successful RNAi response depends on a variety of factors that will be discussed below, including: stability of dsRNA in the gut (affected by gut pH and nucleases), dsRNA length and concentration, target gene, arthropod species and the life-stage exposed (Katoch et al., 2013; Scott et al., 2013; Davis-Vogel et al., 2018; Cooper et al., 2019; Kunte et al., 2020).

Once an insect has ingested dsRNA and the molecule has been taken up by the cells, the endonuclease Dicer cuts the molecule into short interfering RNAs (siRNA) of a length of 20–25 bp that are integrated into the RNA-induced silencing complex (RISC) (Hannon, 2002). Subsequently RISC facilitates the targeting and the endonucleolytic attack on mRNAs with sequence identity to the dsRNA (Hannon, 2002). The pre-requisite for a successful RNAi response is thus sequence identity between at least some of the siRNAs derived from the dsRNA and the target mRNA of the insect pest (Scott et al., 2013). Consequently, length of the dsRNA affects the effectiveness of the RNAi response, as longer molecules yield larger populations of overlapping siRNA molecules ranging in size and sequence (Baum et al., 2007; Bolognesi et al., 2012; Miller et al., 2012; Li et al., 2015b; Nandety et al., 2015). An injection study with *Tribolium castaneum* (Coleoptera: Tenebrionidae) suggests that the size of the dsRNA molecule also affects the duration of the RNAi response, event though the mechanism involved remains unclear (Miller et al., 2012). There is evidence that contiguous sequence matches of $\geq$21 nt of the dsRNA to the target gene are necessary for dsRNA to be biologically active in insects (Bachman et al., 2013, 2016; Roberts et al., 2015) and it has been reported that even a single 21 nt sequence match can induce effects (Bolognesi et al., 2012). It has to be noted, however, that RNAi has been demonstrated to occur at sequence length as short as 15 bp (Powell et al., 2017). Still uncertain is the extent of sequence mismatch that has to be present in order to prevent dsRNA-derived siRNAs. Because siRNA molecules can inhibit translation of transcripts even when mismatches occur, the threshold for concern about non-target effects could be less than 100% sequence identity (Scott et al., 2013). For providing the evidence that any observed effect is due to specific gene silencing, it is necessary to support the feeding assays by determination of transcript levels with RT-qPCR. This, however, poses the challenge of identifying suitable reference or housekeeping genes to calculate relative transcript levels. Furthermore, the effect of RNAi on the protein may not be well correlated to the level of transcript suppression (Scott et al., 2013).

While functional RNAi has been reported from a number of insect species belonging to various orders, the impact of dietary RNAi is more limited (Baum and Roberts, 2014). While many insects have been found to be susceptible to dietary RNAi (Belles, 2010), large differences in sensitivity have been reported across taxa (Whangbo and Hunter, 2008; Terenius et al., 2011; Cooper et al., 2019). For example, feeding studies where solutions containing dsRNA were provided demonstrated that many Coleoptera show a LC_{50} at dsRNA concentrations from 1 to −10 ppb, while effects are seen in Diptera at 10–500 ppm, and in Lepidoptera/Hemiptera at > 1000 ppm (Baum and Roberts, 2014). It has to be noted, however, that sensitivity to dietary RNAi can vary significantly among even closely related species as has been demonstrated for sweetpotato weevils, *Cylas* spp. (Coleoptera: Brentidae) (Christiaens et al., 2016; Prentice et al., 2017). It can even vary between strains/populations of a particular species as has for example been reported for *Locusta migratoria*

(Orthoptera: Acrididae) (Sugahara et al., 2017) and *T. castaneum* (Kitzmann et al., 2013; Spit et al., 2017).

Degradation of the dsRNA after ingestion or uptake is a major factor affecting the exposure of non-target species to bioactive dsRNA molecules and thus the effectivity of RNAi (Wang et al., 2016). Gut pH is important as it affects the stability of the ingested dsRNA molecules. Since RNA is most stable at pH of 4.0–5.0, the slightly acidic midguts of Coleoptera and Hemiptera (pH around 5) support dsRNA stability. In contrast, stability is low in the alkaline guts of Orthoptera, Diptera and Hymenoptera and in particular in the highly alkaline guts of Lepidoptera (pH > 8.0) (Cooper et al., 2019). In addition, dsRNA can be degraded by nucleases in the insect guts as has for example been reported for *Bombyx mori* (Lepidoptera: Bombycidae) (Arimatsu et al., 2007; Liu et al., 2012, 2013) and the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) (Wynant et al., 2014). Degradation of dsRNA in the gut also explains the relatively low sensitivity of *Cylas puncticollis* to dietary RNAi when compared to the closely related *C. brunneus* (both Coleoptera: Brentidae) (Christiaens et al., 2016; Prentice et al., 2017). After uptake, dsRNA can be degraded by nucleases in the haemolymph (Wang et al., 2016) as has for example been reported for *Manduca sexta* (Lepidoptera: Sphingidae) (Garbutt et al., 2013) and *A. pisum* (Christiaens et al., 2014).

To enhance the stability of the ingested dsRNA, to prevent degradation by nucleases and to enhance cellular uptake, various carriers have successfully been deployed (Yu et al., 2013; Christiaens et al., 2018b; Kunte et al., 2020; Vogel et al., 2019). This includes lipid-based encapsulations (Whyard et al., 2009; Taning et al., 2016; Lin et al., 2017), cell-penetrating peptides (Gillet et al., 2017), polymers (Zhang et al., 2010; Christiaens et al., 2018a), and other nanoparticles (He et al., 2013; Das et al., 2015). In addition the RNAi response can be enhanced by co-delivery of nuclease-specific dsRNA (Spit et al., 2017; Cooper et al., 2019). Thus, the formulation in which the dsRNA is provided also has to be considered when judging the hazardous potential of the molecule to non-target species.

SELECTION OF TEST SPECIES FOR NON-TARGET STUDIES

Since not all valued non-target arthropods present in the receiving environment that are potentially exposed to the dsRNA-based product can be tested, surrogate (test) species need to be selected for toxicity studies to support the non-target risk assessment. The following description focuses on the selection of test species to detect sequence-specific effects caused by the particular dsRNA molecule under consideration.

Non-target testing of chemical pesticides has a long history in Europe. At the initial stage, only 2 species are tested under worst-case exposure conditions, i.e., the predatory mite *Typhlodromus pyri* (Acari: Phytoseiidae) and the parasitic wasp *Aphidius rhopalosiphi* (Hymenoptera: Braconidae) (Candolfi et al., 2001). The two species were selected as indicators since sensitivity analyses revealed that they are the most sensitive species to most classes of pesticides (Candolfi et al., 1999; Vogt, 2000). Consequently, by testing those species predictions of effects on other non-target arthropods can be made with high confidence (Candolfi et al., 1999). Only if adverse effects above a certain threshold are detected for those species and unacceptable risk can thus not be excluded additional tests with other beneficial species are indicated. These include *Orius laevigatus* (Hemiptera: Anthocoridae), *Chrysoperla carnea* (Neuroptera: Chrysopidae), *Coccinella septempunctata* (Coleoptera: Coccinellidae), and *Aleochara bilineata* (Coleoptera: Staphilinidae). These species were selected because they are commercially available, amenable to testing in the laboratory, reliable test protocols exist, they provide sufficient phylogenetic and functional diversity, and common in agricultural fields (Barrett et al., 1994; Candolfi et al., 2001). In addition to testing predators and parasitoids, most regulatory jurisdictions (e.g., European Commission [EC], 2002), require testing of honey bees (*A. mellifera*) and soil organisms [*Folsomia candida* (Collembola: Isotomidae) or *Hypoaspis aculeifer* (Acari: Gamasidae)], if exposure of the latter is anticipated.

This common set of surrogate test species, however, is not suitable to assess non-target effects caused by dsRNA-based spray products because the initial two indicator species were selected for their sensitivity to chemical pesticides but are unlikely to be the most sensitive species for the majority of dsRNA molecules. Consequently it would be more suitable to apply the approach for non-target risk assessment as is conducted for GE plants expressing insecticidal proteins, such as Bt crops expressing Cry or VIP proteins from *Bacillus thuringiensis*. The ERA for GE plants is conducted case-by-case and consequently the most appropriate non-target species can be selected for each plant/trait combination. It has been proposed to base the selection of test species for laboratory studies on three main criteria (Romeis et al., 2013):

(i) Sensitivity: species should be the most likely to be sensitive to the stressor under consideration based on the known spectrum of activity, its mode of action, and the phylogenetic relatedness of the test and target species.

(ii) Relevance: species should be representative of valued taxa or functional groups that are most likely to be exposed to the stressor in the field. Organisms that contribute to important ecosystem service and are considered relevant have been identified for a number of field crops (e.g., Meissle et al., 2012; Romeis et al., 2014; Riedel et al., 2016; Li et al., 2017).

(iii) Availability and reliability: suitable life-stages of the test species must be obtainable in sufficient quantity and quality, and validated test protocols must be available that allow consistent detection of adverse effects on ecologically relevant parameters. Lists of above-ground, below-ground, and aquatic species that are available and amenable for testing have been published (e.g., Candolfi et al., 2000; Römbke et al., 2010; Romeis et al., 2013; Carstens et al., 2012; Li et al., 2017).

The above listed criteria are also key elements of other test species selection approaches that have for example been published by Todd et al. (2008) and Hilbeck et al. (2014).

While the criteria (ii) and (iii) are relative generic or crop-specific, criteria (i) needs to be addressed specifically for each stressor under consideration. To increase the robustness and

reliability of the non-target risk assessment the species most likely to be sensitive (= affected) to a particular dsRNA should be selected. This includes considerations of the gene or gene family that is targeted and the knowledge about the sensitivity of certain taxa to dietary RNAi in general. The phylogenetic relationship of the non-target organisms to the target pest should also be considered, as there is evidence that, in general, species closely related to the target organism are more likely to be susceptible to the dsRNA than distantly related species (Whyard et al., 2009; Bachman et al., 2013, 2016; US EPA, 2014; Roberts et al., 2015).

Since the RNAi response is sequence specific, bioinformatics can help predicting the species most likely affected that could then be used in feeding studies (Bachman et al., 2013, 2016). However, it has to be recognized that the presence of sequence homologies between the dsRNA molecule and the genome of the non-target species does not necessarily indicate sensitivity of an organisms. For example, the springtail *Sinella curviseta* (Collembola: Entomobryidae) shares a total of six 21 nt long matches with the dsRNA targeting the *vATPase A* in *D. v. virgifera*. However, the organism was not adversely affected in laboratory feeding studies (Pan et al., 2016). In cases where for some reason (species that are rare, protected or difficult to rear), bioinformatics may, however, be the only way to "test" the species (Bachman et al., 2016). Bioinformatics could also help predicting off-target effects. However, currently we lack genomic data for most non-target species. It would be useful to have more genome data available for model non-target species that actually play a role in agricultural production systems to effectively apply bioinformatics to the NTO risk assessment (Casacuberta et al., 2015; Fletcher et al., 2020).

DESIGN AND IMPLEMENTATION OF NON-TARGET LABORATORY TOXICITY STUDIES

The established test protocols published by the West Palaearctic Regional Section of the International Society for Biological and Integrated Control (IOBC/WPRS; Candolfi et al., 2000) or by the European and Mediterranean Plant Protection Organization (EPPO)[2] for early-tier laboratory toxicity studies for chemical insecticides are based on contact toxicity. Those test protocols thus do not allow assessing the non-target effects of dsRNA for which oral uptake is the most important route of exposure. The lack of standardized test protocols addressing the oral route of exposure and to detect effects resulting from novel modes of action has recently been pointed out by the Panel on Plant Protection Products and their Residues of European Food Safety Authority (2015) even though RNAi was not specifically mentioned.

However, experience is available with gut-active insecticidal proteins such as the Cry and VIP proteins from *B. thuringiensis*. Guidance exists on how to design and perform laboratory feeding studies with such proteins to provide high quality, reliable and robust data (Romeis et al., 2011; De Schrijver et al., 2016). When designing a non-target laboratory study the following main criteria should be considered (Romeis et al., 2011): (i) Test substance characterization and formulation; (ii) Method of delivery; (iii) Concentration/dose; (iv) Measurement endpoints; (v) Test duration; (vi) Control treatments; (vii) Statistical considerations.

Since the formulation in which the dsRNA is provided has a strong effect on the dsRNA uptake and the strength of the RNAi response in arthropods (as discussed above) care should be taken that the test substance is provided in a realistic formulation.

It is generally considered that toxicity of insecticidal compounds such as chemical insecticides and Cry proteins from Bt increases with increasing concentration in which they are delivered. Thus safety is added to the non-target studies by testing unrealistically high concentrations of the stressor of concern to provide a margin of safety and to account for possible intra- and interspecific variability from the use of a surrogate test species. Definition of the concentrations to be tested poses some challenges for different reasons. First, the length of the dsRNA affects the effectiveness to trigger an RNAi response (Bolognesi et al., 2012; Miller et al., 2012), thus the margins of safety may vary between constructs. Second, there is evidence that there is no clear dose-relationship but that RNAi is triggered from a specific threshold dose onward and might be maximal at an optimal dose (Turner et al., 2006; Niu et al., 2019). Third, high doses may cause sequence-unspecific effects as discussed above.

The endpoints to be recorded (lethal and sublethal) need to be selected based on the organism under investigation (and the reliability of the test system) and the gene that is targeted. While lethality is an obvious endpoint to be chosen, the consideration of sublethal endpoints such as growth or development time is recommended (Roberts et al., 2020). First, they may hint to unexpected off-target effects, second, they may cover for the fact that dsRNA is generally slow acting (Baum and Roberts, 2014) and that the process is typically not reaching 100% gene suppression (e.g., Bolognesi et al., 2012; Rangasamy and Siegfried, 2012), and third, they might address the fact that RNAi effects can be transgenerational, i.e., also affecting subsequent generations (Abdellatef et al., 2015). Sublethal endpoints are typically also recorded in the testing of chemical pesticides (e.g., Candolfi et al., 2000) and Bt proteins (De Schrijver et al., 2016; Roberts et al., 2020) even though mortality is the primary endpoint and often the results from testing sublethal endpoints are not reported in regulatory summaries. In any case, it is important to set decision-making criteria for every endpoint that is recorded. The duration of the study needs to be selected so that the measurement endpoints show a response should the test substance have an effect. Given the slow RNAi response, test probably need to be extended in duration compared to Bt Cry proteins (e.g., Bachman et al., 2013, 2016).

A key element of every laboratory study is the inclusion of a negative control treatment that allows to separate effects caused by the test system (e.g., the fitness of the test organisms, the suitability of the diet) from those caused by the test substance. Ideally, the negative control consists of a dsRNA molecule that targets a heterologous sequence absent from the insect's genome and that does thus not lead to specific gene silencing

[2]https://pp1.eppo.int/standards/side_effects

in the test species. This would control for any impact caused by a trigger of the RNAi cascade (sequence unspecific effects). Typical examples that have been used for this purpose include dsRNA targeting the green fluorescent protein (GFP) and β-glucuronidase (GUS). However, there is some evidence, that dsGFP causes adverse effects in arthropods when applied orally at very high doses (Nunes et al., 2013; Haller et al., 2019) or when injected (Jarosch and Moritz, 2012).

Positive controls, i.e., the addition of dsRNA molecules that are designed to silence a gene in the test insects can further help to interpret the study results as they provide evidence that the test system can detect a response and that the test species is sensitive to dietary RNAi. Positive controls have for example been deployed by Haller et al. (2019) when testing the effect of dsRNA targeting the *vATPase-A* of *D. v. virgifera* in two non-target ladybird beetles (Coleoptera: Coccinellidae). The data confirmed that two species of ladybirds are sensitive to dietary RNAi but that the non-target dsRNA molecule only had a weak effect. Another study using the same test substance in honey bees did not detect any effects in the positive control treatment raising doubts about the sensitivity of honey bees to dietary RNAi in general (Vélez et al., 2016).

CONCLUSION

In order to assess whether dsRNA-based pesticide sprays adversely affect valued non-target species in the agroecosystem, three questions need to be addressed: (1) Are the non-target arthropods exposed to biologically active dsRNA? (2) Do the non-target arthropods possess the RNAi machinery for dsRNA to trigger a response? and (3) are there sufficient sequence matches between the dsRNA molecule under consideration and the genome of the non-target arthropods to cause a sequence-specific effect.

While it is possible to make some generalizations regarding the level of exposure, potential uptake of dsRNA and the sensitivity to dietary RNAi for common non-target species in field crops, some open questions remain. For example it is still unclear to what extent the bioactive dsRNA molecule is transferred through the arthropod foodweb and whether penetration through the arthropod body wall is a relevant route of exposure for non-target species. Furthermore, it would be useful to evaluate whether the risk for certain arthropod taxa can be considered negligible because they digest dsRNA prior to ingestion and are thus unlikely to be exposed.

Concerning the hazard posed by dsRNA, it would be important to evaluate whether there are species or taxa that can be considered safe because they are insensitive to dietary RNAi in general (e.g., because they lack the dsRNA uptake mechanism). Also, uncertainty still exists regarding the sequence mismatches (and number thereof) between the targeted mRNA and the dsRNA that still allows for an RNAi response. There is evidence that genome information can help assess non-target effects. However, bioinformatics information is still lacking for most valued non-target arthropods. This information would help assist to predict non-target effects and select the most suitable (i.e., potentially sensitive) species to conduct feeding studies in the laboratory. Related to this, the power of bioinformatics for predicting non-target effects still needs to be further investigated before this information can be used to draw a conclusion about safety.

Consequently, it is essential to conduct feeding studies to assess whether the ingestion of dsRNA molecules poses a hazard to relevant non-target species. However, when planning the studies to be conducted in the laboratory with dsRNA-based pesticides, it would be necessary to add flexibility to the non-target risk assessment framework used for chemical pesticides to allow a case-by-case assessment as is done for GE plants. A challenge remains the selection of the most appropriate negative and positive control treatments to ensure a robust interpretation of the study results and to minimize false negative and false positive results.

The main concern, however, is the fact that the carrier to which the dsRNA is bound or the formulation in which it is applied will be of ample importance as it not only affects the level at which non-target arthropods will be exposed, i.e., the stability and distribution of the active compound in the environment and in the insect gut and body, but also the extent of the RNAi response.

While there is a lot to profit from the experience with chemical pesticides and GE plants producing insecticidal proteins, insecticidal sprays based on dsRNA still pose some specific challenges to the non-target risk assessment.

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REVIEW
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Key Mechanistic Principles and Considerations Concerning RNA Interference

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Canonical RNAi, one of the so-called RNA-silencing mechanisms, is defined as sequence-specific RNA degradation induced by long double-stranded RNA (dsRNA). RNAi occurs in four basic steps: (i) processing of long dsRNA by RNase III Dicer into small interfering RNA (siRNA) duplexes, (ii) loading of one of the siRNA strands on an Argonaute protein possessing endonucleolytic activity, (iii) target recognition through siRNA basepairing, and (iv) cleavage of the target by the Argonaute's endonucleolytic activity. This basic pathway diversified and blended with other RNA silencing pathways employing small RNAs. In some organisms, RNAi is extended by an amplification loop employing an RNA-dependent RNA polymerase, which generates secondary siRNAs from targets of primary siRNAs. Given the high specificity of RNAi and its presence in invertebrates, it offers an opportunity for highly selective pest control. The aim of this text is to provide an introductory overview of key mechanistic aspects of RNA interference for understanding its potential and constraints for its use in pest control.

Keywords: RNAi, dicer, argonaute, miRNA, dsRNA, off-targeting

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INTRODUCTION

RNA interference (RNAi) is one of the pathways, collectively named RNA silencing pathways, that employ small RNAs as guides for sequence-specific silencing [reviewed in (Ketting, 2011)]. RNAi was discovered in *C. elegans* and defined as sequence-specific mRNA degradation induced by long double-stranded RNA (dsRNA) (Fire et al., 1998). Although some authors use the term RNAi as a synonym for RNA silencing [e.g., (Ketting, 2011)], this review will adhere to the original definition as formulated by Fire et al.

The primary aim of this contribution is to provide an overview of RNA interference mechanism with focus on selected aspects concerning RNAi targeting and off-targeting in animals as these would be most relevant features for discussing the use of RNAi for pest control. Therefore, I will

purposefully not go into the details. Interested readers should check out referenced reviews or original articles. For a thorough overview of RNAi, readers are welcome to refer to a comprehensive compilation of information on RNAi and related pathways in different animal taxons and plants, which we assembled with colleagues for the European Food and Safety Authority (Paces et al., 2017).

PRINCIPLES OF RNA SILENCING AND COMMON DENOMINATORS

Some kind of RNA silencing pathway (**Figure 1A**) exists in almost every eukaryotic organism with some notable exceptions among fungi and protists (Nakayashiki et al., 2006; Matveyev et al., 2017). RNA silencing pathways utilize 20-30 nucleotide long RNAs loaded on Argonaute proteins, which guide sequence-specific repression through basepairing with target RNAs. RNA silencing pathways differ in the origin and biogenesis of small RNAs, mechanisms leading to target repression, and biological roles [reviewed in (Ketting, 2011)].

RNA substrates giving rise to small RNA guides in RNA silencing pathways vary in structure. They include double-stranded RNA (dsRNA) with blunt ends, small and long RNA hairpins with perfect and less-than-perfect complementarity, sense and antisense RNA (basepaired or not), or single-stranded "aberrant" RNA that would be converted to dsRNA by an RNA-dependent RNA polymerases (RdRP) or converted directly to small RNAs. Substrates can be converted to a small RNA either by Dicer, an RNase III cleaving dsRNA and/or canonical microRNA (miRNA) precursors, or by some Dicer-independent mechanism [reviewed in (Kim et al., 2009)].

Target repression can be post-transcriptional or transcriptional. Post-transcriptional RNA silencing could have a form of endonucleolytic cleavage of cognate RNA (traditionally associated with RNAi), or translational repression coupled with mRNA destabilization (historically associated with animal miRNAs). Transcriptional RNA silencing is common in plants but rare among animals [reviewed in (Wassenegger, 2005; Malik and Svoboda, 2012)]. It may involve *de novo* DNA methylation or transcriptionally repressive histone modifications.

Common biological roles of RNA silencing pathways include regulation of endogenous gene expression, antiviral immunity, and genome protection against transposable elements [summarized in (Ketting, 2011)]. During evolution, RNA silencing could evolve into a complex system of interconnected pathways [exemplified by plants, reviewed for example in (Borges and Martienssen, 2015)] or into a relatively simple set up (mammalian soma). The following text will focus on RNAi but includes also the miRNA pathway because of its close mechanistic relationship to RNAi.

RNAi PATHWAY

The canonical RNAi pathway (**Figure 1B**) is initiated by cleavage of long dsRNA into small interfering RNAs (siRNAs). One siRNA strand then becomes loaded onto an Argonaute protein possessing endonucleolytic activity (e.g., AGO2 in vertebrates and arthropods). A complementary mRNA is cleaved by the Argonaute in the middle of the siRNA:mRNA duplex. In some taxons (e.g., plants or *C. elegans*), RNAi pathways employ the above-mentioned RdRPs, which can provide an amplification loop synthesizing small RNAs or dsRNA on targeted RNA templates [reviewed in (Maida and Masutomi, 2011)]. *C. elegans* employs so-called "transitive RNAi" where RdRP produces secondary siRNAs extending upstream of the targeted sequence (Sijen et al., 2001). Plants also exhibit transitive silencing (Vaistij et al., 2002); the transitivity may even spread downstream of the targeted sequence (Moissiard et al., 2007).

Canonical RNAi is traditionally viewed as a defense pathway providing antiviral innate immunity in invertebrates and plants against viruses that produce dsRNA (Ding and Voinnet, 2007). However, RNAi could evolve additional roles, such as maintenance of genome integrity through suppression of

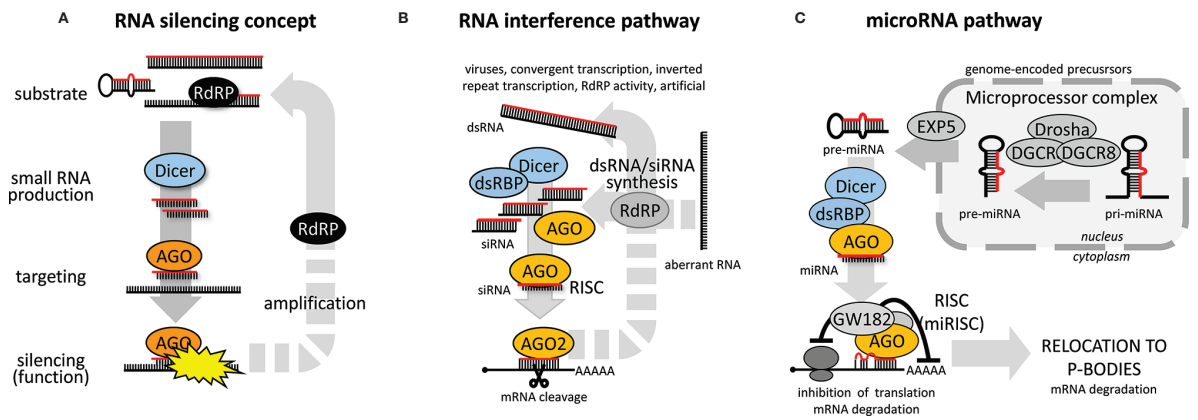

FIGURE 1 | RNA silencing pathways. **(A)** General concept of RNA silencing. **(B)** General RNAi pathway overview, and **(C)** miRNA pathway (animal set up).

transposable elements or control of gene expression. In plants, for example, the basic RNAi mechanism has been integrated into a complex pathway system of post-transcriptional and transcriptional silencing, which employs multiple Dicer, Argonaute and RdRP proteins and functions in antiviral defense, protection of genome integrity, and regulation of gene expression [reviewed for example in (Bologna and Voinnet, 2014; Borges and Martienssen, 2015)]. In *C. elegans*. RNAi exists as a complex of antiviral RNAi, endo-RNAi controlling endogenous genes, and exo-RNAi responding to dsRNA in the environment [reviewed in (Billi et al., 2014)]. RNAi is functional in insects (Dowling et al., 2016) and other arthropod subphyla, including *Chelicerata* [ticks and mites (Kurscheid et al., 2009; Schnettler et al., 2014; Hoy et al., 2016)] and *Crustacea* [shrimps (Chen et al., 2011; Huang and Zhang, 2013; Yang et al., 2014)]; genomic data suggest that *Myriapoda* arthropods also have functional RNAi (Palmer and Jiggins, 2015). In vertebrates, the RNAi pathway has become vestigial; protein factors for siRNA biogenesis and target repression serve the miRNA pathway [reviewed in (Svoboda, 2014)]. This is presumably a consequence of the innate immunity system evolving an array of protein sensors detecting pathogen markers such as dsRNA, which trigger the so-called interferon response [reviewed in (Gantier and Williams, 2007)]. An important limiting factor for functional RNAi in somatic mammalian cells seems to be inefficient siRNA production due to the low processivity of mammalian Dicer, which is adapted for non-processive miRNA biogenesis (Demeter et al., 2019).

miRNA PATHWAY

While the miRNA pathway (**Figure 1C**) can share some components with the RNAi pathway, it differs in several fundamental aspects. miRNAs are genome-encoded repressors of gene expression with defined sequences (i.e., can be precisely annotated). While RNAi employs a population of siRNAs stochastically generated from dsRNA to destroy a pool of RNAs with the complementary sequence, one specific miRNA sequence can guide repression of many different mRNAs through imperfect miRNA:mRNA basepairing.

Animal miRNA biogenesis [reviewed in (Kim et al., 2009)] starts with a primary miRNA (pri-miRNA), a long Pol II transcript carrying one or more local hairpins, which can be cut out from the pri-miRNA by RNase III activity of the nuclear Microprocessor complex. The resulting miRNA precursor (pre-miRNA) is transported to the cytoplasm, where it is cleaved by Dicer. One strand of the resulting duplex is loaded onto an AGO protein similarly to the RNAi pathway. Vertebrates have usually four AGO paralogs; teleost fish acquired an additional AGO3 paralogue through a fish-specific genome duplication event (Mcfarlane et al., 2011). All four mammalian AGO proteins accommodate miRNAs equally well (Meister et al., 2004), including AGO2, which is the only one with "slicing" endonucleolytic activity. All four mouse AGO proteins seem to be functionally redundant in the miRNA pathway, as shown by rescue experiments in embryonic stem cells lacking all four *Ago* genes (Su et al., 2009).

Typical miRNA:mRNA interaction in animals occurs with partial complementarity (described in detail further below) and results in translational repression, which is associated with substantial mRNA degradation. Plant miRNA biogenesis [reviewed in (Jones-Rhoades et al., 2006)] employs one of the Dicer paralogs (DCL1), which processes both pri-miRNA and pre miRNA. Plant miRNAs often have higher sequence complementarity resulting in RNAi-like cleavage of their targets but also frequently repress translation (Brodersen et al., 2008; Lanet et al., 2009). In animals, miRNAs can also mediate RNAi-like cleavage, as demonstrated by reporters designed to have full complementarity to a specific miRNA (Schmitter et al., 2006), but naturally occurring RNAi-like endonucleolytic cleavage of targets is rare (Yekta et al., 2004). The experimental approach to knocking down gene expression in mammalian cells by delivering a siRNA (either as an *in vitro* synthesized RNA or expressed from a plasmid vector) is commonly called RNAi. Mechanistically, however, the approach hijacks the miRNA pathway and its aforementioned ability to produce RNAi-like cleavage.

CO-EXISTENCE OF RNAi AND miRNA PATHWAYS

While there is an apparent mechanistic overlap, there is functional divergence of RNAi and miRNA pathways, which likely influenced the co-existence of the two pathways in different model systems during evolution (**Figure 2**). One is represented by *Drosophila*, where both pathways genetically diverged such that each pathway has a dedicated Dicer and AGO protein, while the crosstalk between the two pathways is minimal. Dicer in the RNAi pathway is phylogenetically more derived, which would be consistent with its engagement in dsRNA-based antiviral defense and host-pathogen evolutionary arms race (Murphy et al., 2008; Obbard et al., 2009). *C. elegans* employs a single Dicer in production of miRNAs and siRNAs, but has a complex system of Argonaute proteins and RdRP amplification, which contributes to the separation of the pathways. Mammals have a single Dicer mainly serving for miRNA biogenesis; canonical RNAi was functionally replaced by the interferon response, which allows for sensing more structural features of replicating RNA viruses. Functional RNAi in mammalian cells requires high Dicer activity, enough dsRNA substrate, and suppression of the interferon response (Kennedy et al., 2015; Maillard et al., 2016; Kennedy et al., 2017; Van Der Veen et al., 2018; Demeter et al., 2019). However, these three conditions are rarely met—a unique example occurs in the mouse oocyte [reviewed in (Svoboda, 2014)].

Interestingly, in one of the plant RNA silencing mechanisms, RNAi essentially serves as an amplifier of miRNA silencing where miRNA-mediated cleavage of mRNA targets is followed by RdRP-mediated production of long dsRNA, which is processed by Dicer into so-called phased siRNAs (phasiRNA).

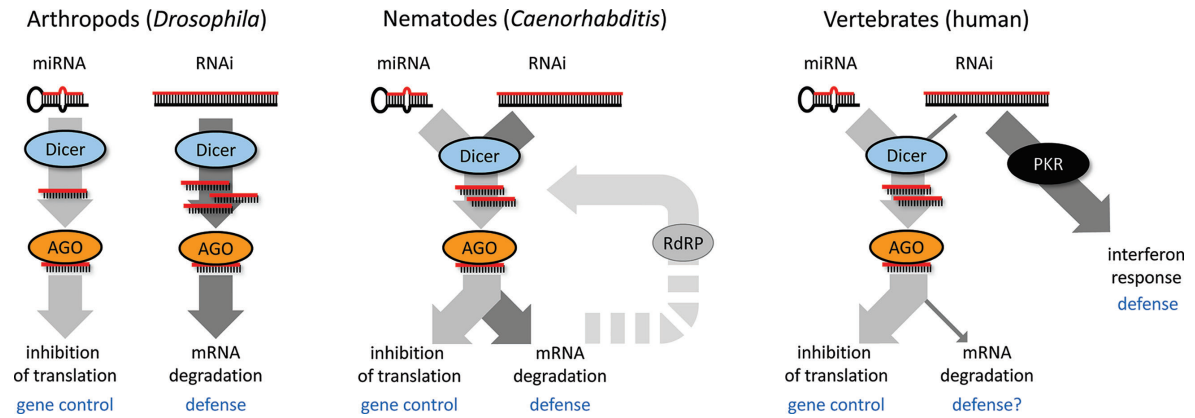

FIGURE 2 | Different scenarios of co-existence of RNAi and microRNA (miRNA) in different species.

PhasiRNAs themselves are a complex small RNA category as they can be generated by different Dicers and mediate target cleavage as well as transcriptional silencing. (reviewed in (Komiya, 2017; Deng, 2018).

IMPORTANT MECHANISTIC DETAILS OF RNAi

Substrate Processing by Dicer and Types of Small RNA Populations

RNase III Dicer (reviewed in detail in [Jaskiewicz and Filipowicz, 2008; Svobodova et al., 2016)] is the enzyme producing small RNAs in canonical RNAi and miRNA pathways. Dicer is a large (~200 kDa) multidomain protein (**Figure 3A**). Structural and biochemical analyses (mainly in mammals but also in the protozoan *Giardia intestinalis*) uncovered how canonical Dicer generates small RNAs of defined length from long dsRNA substrates (Provost et al., 2002; Zhang et al., 2002; Zhang et al., 2004; Macrae et al., 2006; Macrae et al., 2007). Dicer preferentially cleaves dsRNA at the termini (**Figure 3B**). A dsRNA terminus is bound by the PAZ domain, which has high affinity to 3' protruding overhangs, typical termini of canonical miRNA precursors and of processive cleavage of long dsRNA (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Ma et al., 2004). A canonical Dicer functions as a molecular ruler defining the length of a small RNA by the distance between the PAZ domain and RNase III cleavage sites (Macrae et al., 2006). Dicer has two RNase III domains, which form a single processing center containing two catalytic "half sites" (Zhang et al., 2004; Macrae et al., 2006). Each of them cleaves one strand of the dsRNA, producing a small RNA duplex with two nucleotide 3' overhangs and 5' monophosphate and 3' hydroxyl groups at the RNA termini (Zhang et al., 2004). The length of the product depends on the specific Dicer. A typical length of an animal Dicer product is 22 nucleotides although 20-22 nt siRNAs was reported for different insects (Santos et al., 2019). *Giardia* produces 25 nt small RNAs, plants, which utilize several Dicer paralogs (**Figure 3A**), produce shorter (21/22 nt) and longer (24 nt) small RNAs (Jaskiewicz and Filipowicz, 2008).

Dicer can process structurally different dsRNA substrates—e.g., small hairpins of pre-miRNAs, dsRNA with blunt ends, or dsRNA with long single-strand overhangs or loops (**Figure 3C**). As mentioned above, Dicer structure implies that Dicer preferentially cleaves dsRNA at the termini. However, as shown for human Dicer, it can also cleave the dsRNA stem internally, albeit with low efficiency (Provost et al., 2002; Zhang et al., 2002). The type of dsRNA processing determines the composition of a small RNA population produced from each type of the template (**Figure 3C**). miRNAs are precisely defined because precursors have a uniform structure and there is just a single Dicer cleavage event. Long blunt-end dsRNA, which is cleaved processively from its ends, generates phased siRNAs produced by consecutive cleavage. In this case, there may be some variability/shifts as the termini are not as precisely defined as 2nt overhangs of miRNA precursors. Dicers with low processivity, exemplified by mammalian Dicers, generate siRNAs mainly from dsRNA termini—RNAi efficiency in this case thus depends on the efficiency of the first siRNAs at the termini (Demeter et al., 2019). When Dicer cannot initiate cleavage from a terminus because it is, for example obstructed by longer overhangs, dsRNA processing is initiated by an internal cleavage; the resulting siRNA population appear random and there would be no evidence of phasing [e.g., (Tam et al., 2008; Watanabe et al., 2008)].

Loading—Small RNA Sorting Onto Argonaute Proteins

Loading of a small RNA onto an Argonaute protein is the key step in formation of the RNAi effector complex also known as RNA-induced silencing complex (RISC). While Argonaute proteins interact with many other proteins [reviewed in (Meister, 2013)], the minimal RNAi effector complex, the holo-RISC, is a specific Argonaute loaded with a siRNA. Loading is an important step for selecting the targeting strand and sorting small RNAs into distinct RNA silencing pathways. As shown for

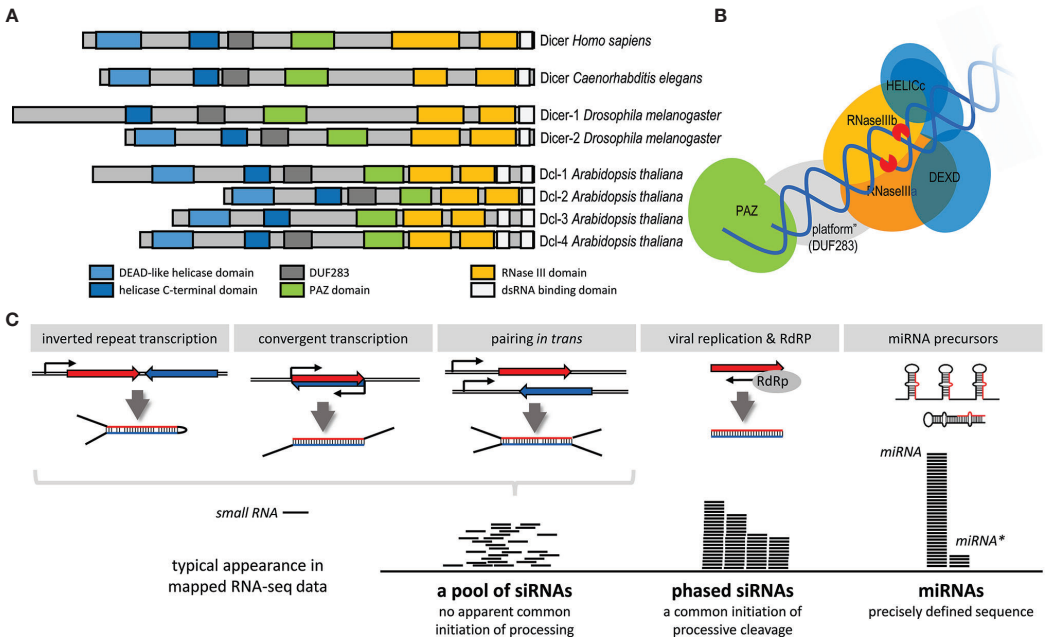

FIGURE 3 | Dicer and production of small RNAs. **(A)** Domain composition of Dicer proteins in a common multicellular model system. **(B)** Schematic organization of Dicer and its interaction with dsRNA [based on the mammalian Dicer structure (Lau et al., 2012)]. **(C)** Model examples of different types of Dicer substrates and products. Production of phased small interfering RNAs (siRNAs) requires a double-stranded (dsRNA) terminus where Dicer will initiate processive cleavage. It could be produced by an RdRP, typically during viral replication. In specific cases, such as plant phased siRNAs (phasiRNA), also by a cellular RdRp [reviewed in (Komiya, 2017)]. However, not all RdRP-produced dsRNAs result in the formation of phased RNAs.

animal Argonautes, loading a specific strand of the small RNA duplex produced by Dicer, exhibits a thermodynamic bias where the strand whose 5′-end is less thermodynamically stable is preferentially loaded onto AGO as the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). This feature is important for designing effective siRNAs for experimental repression.

Loading is assisted by a family of proteins with tandemly organized dsRNA binding domains (dsRBDs), which interact with Dicer and AGO proteins to form the RISC loading complex (RLC). Sorting through RLC varies among animal taxons. For example, *C. elegans* employs a single Dicer protein, but evolved an extreme diversity of Argonaute proteins among common model systems [25-27 Argonaute family members (Buck and Blaxter, 2013)]. Together with RdRPs, RNA silencing in *C. elegans* is a complex system of biogenesis and sorting of primary and secondary cytoplasmic and nuclear small RNAs in soma and germline (Yigit et al., 2006; Buck and Blaxter, 2013). The exo-RNAi pathway in *C. elegans* involves loading of AGO protein RDE-1 with primary siRNAs with the assistance of dsRBP RDE-4 (Tabara et al., 1999; Parrish and Fire, 2001; Tabara et al., 2002; Lu et al., 2005; Wilkins et al., 2005). This is followed by biogenesis of secondary siRNAs (22G RNAs) loaded on AGO protein CSR-1 (Aoki et al., 2007). *C. elegans* miRNAs are exclusively loaded on ALG-1/2 AGO proteins (Correa et al., 2010). *Drosophila* employs dedicated Dicer and Argonaute proteins for RNAi (DCR-2 and AGO2) and miRNA pathways (DCR-1 and AGO1). Loading of each AGO is assisted by two dsRBPs: R2D2 [its orthologs exist in winged insects (Dowling et al., 2016)] is coupled with the RNAi pathways and Loquacious (LOQS) primarily with the miRNA pathway; these two dsRBPs thus bridge processing of specific substrates by both Dicers and their loading onto specific AGO proteins, although the separation is not complete (Forstemann et al., 2007; Tomari et al., 2007; Okamura et al., 2008; Czech et al., 2009; Okamura et al., 2009; Ghildiyal et al., 2010). Mammals, in contrast, have minimal if any sorting of small RNAs and load them onto all four AGO proteins equally well (Meister et al., 2004; Burroughs et al., 2011; Dueck et al., 2012). This is presumably because the mammalian RNAi pathway is vestigial and the silencing machinery primarily serves the miRNA pathway.

Targeting—The Seed Sequence

Recognition of targets is coupled with the loaded Argonaute structure (**Figures 4A, B**). The human AGO2 has a bilobed composition with a central cleft for binding guide and target RNAs (Elkayam et al., 2012; Schirle and Macrae, 2012; Schirle et al., 2014; Schirle et al., 2015). AGO2 binds both ends of a siRNA. The 5' end is buried in a pocket between MID and PIWI domains, while the 3' end is anchored in the PAZ domain (Ma et al., 2004). The PIWI domain has an RNase H-like fold and provides the endonucleolytic "slicer" activity (Song et al., 2004; Yuan et al., 2005).

A small RNA loaded onto an animal AGO protein has five distinct sequence modules: the anchor, seed, central, 3'

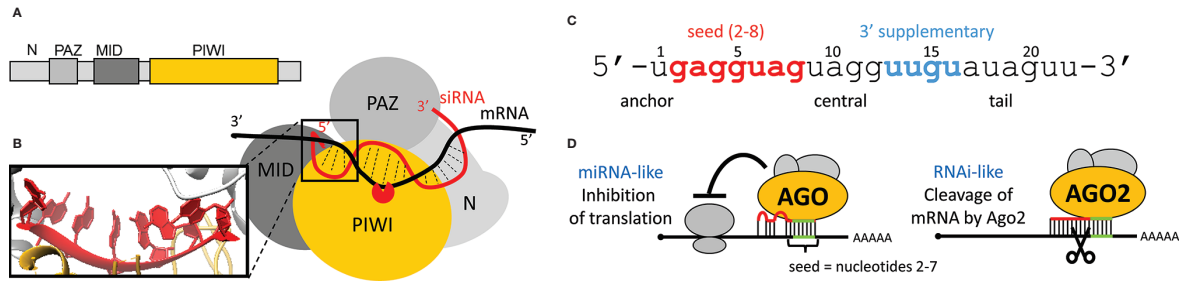

FIGURE 4 | Argonaute protein and target repression. **(A)** Domain composition of human AGO2. **(B)** Schematic organization of domains in AGO2. Magnified is the 5' end of a small RNA and its A-like form. **(C)** Division of a small RNA into five different modules as described by Wee at al. (Wee et al., 2012). **(D)** Schematic depiction of miRNA-like and RNAi-like silencing effects. An RNAi-like effect requires extensive sequence complementarity and AGO2.

supplementary, and tail (**Figure 4C**) (Wee et al., 2012). The 5' end nucleotides 2 to 6 are positioned in an A-form (**Figure 4B** -inset) conformation facilitating basepairing with the target (Schirle and Macrae, 2012). Structural analysis of the human AGO2 suggested a stepwise mechanism for interaction with cognate RNAs, where AGO2 exposes nucleotides 2 to 5 for initial target pairing, which then promotes conformational changes that expose nucleotides 2 to 8 and 13 to 16 for further target recognition (Schirle et al., 2014). Structural data were corroborated by kinetic data and single molecule analyses, which support the idea that different regions of the siRNA play distinct roles in the cycle of target recognition, cleavage, and product release (Haley and Zamore, 2004; Li et al., 2012; Wee et al., 2012; Zander et al., 2014; Salomon et al., 2015). The seed sequence disproportionately contributes to target RNA-binding energy, whereas base pairs formed by the central and 3' regions of the siRNA provide a helical geometry required for catalysis (Haley and Zamore, 2004). Because of the A conformation of the seed, a loaded AGO2 exhibits kinetic properties more typical of RNA-binding proteins and does not follow the rules by which sole oligonucleotides find, bind, and dissociate from complementary nucleic acid sequences (Salomon et al., 2015). Importantly, the concept of the seed sequence is fundamental for understanding one of the main causes of off-targeting.

Targeting—Complementarity and Cleavage

For the Argonaute function in RNAi, a "two-state" model was proposed (Tomari and Zamore, 2005), where the seed guides binding to the target, while pairing of the 3' end requires dislodging of the 3' end from the PAZ domain in order to cleave the cognate RNA. Efficient cleavage requires full complementarity in the middle of the basepaired sequence, in order to be cleaved by the PIWI domain (**Figure 4B**). Mismatches in the central part of the small RNA interfere with the cleavage and explain the high specificity of RNAi (i.e., endonucleolytic cleavage by the AGO2 slicer activity) (**Figure 4D**).

Single-molecule experiments with the loaded AGO2 showed that target binding starts at the seed region of the small RNA (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). AGO2 initially scans for target sites with complementarity to nucleotides 2–4 of the miRNA. This initial interaction propagates into stable association when the target complementarity extends to nucleotides 2–8. The recognition process is coupled to lateral diffusion of AGO2 along the target RNA, which promotes the target search by enhancing the retention of AGO2 on the RNA (Chandradoss et al., 2015). RISC binding with the seed match can thus be established, which is consistent with the seed-match rule of miRNA target selection (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). An important conclusion from the kinetic analysis by Wee et al. is that low-abundant miRNAs are unlikely to contribute biologically meaningful regulations, because they are present at concentrations below their K_D for seed-matching targets, which are in a picomolar range (3.7 pM for mouse AGO2 and 20 pM for *Drosophila* AGO2) (Wee et al., 2012). Importantly, accessibility of the target for seed sequence binding is another important factor for efficient targeting. It was shown that the accessibility of the target site correlates directly with the efficiency of cleavage, recognition of inaccessible sequences is impaired because RISC does not unfold structured RNA (Ameres et al., 2007).

siRNA-mediated target recognition is highly specific. However, discrimination of RNAi between two sequences differing by a single nucleotide depends on the position and type of the mismatch (Du et al., 2005; Holen et al., 2005; Haley et al., 2010). Analysis of minimal siRNA complementarity in *Drosophila* showed that perfect complementarity at positions 2–17 is sufficient for RNAi (Haley et al., 2010). G:U wobble basepairs are surprisingly well tolerated; target sites containing such mismatches were silenced almost as efficiently as with full complementarity (Du et al., 2005). Tolerated can also be A:C mismatches (Du et al., 2005).

Of note is that consensus basepairing rules for functional plant miRNA-target interactions differ from those for animals: there is little tolerance of mismatches at nucleotides 2–13, with especially little tolerance of mismatches at nucleotides 9–11, and more tolerance of mismatches at nucleotides 1 and 14–21 (Wang et al., 2015). Furthermore, the perfect complementarity is not as prevalent as usually thought among plant miRNAs, as most of the identified miRNA targets in plant cells have some imperfect basepairing [summarized in (Jones-Rhoades et al., 2006)].

RdRP Enhancer of RNAi—Transitive RNAi

RdRPs can contribute to RNAi by converting single-stranded RNA to dsRNA or by synthesizing short RNAs that could be loaded onto AGO proteins. Importantly, all RdRPs identified so far seem to come from one ancestral RdRP, whose orthologs were found in plants, fungi and some animals (Cerutti and Casas-Mollano, 2006; Murphy et al., 2008). Homologs of RdRPs exist in numerous metazoan taxons, including *Nematoda* (e.g., *Caenorhabditis elegans*), *Cnidaria* (hydra), *Chelicerata* (tick), *Hemichordata* (acorn worm), *Urochordata* (sea squirt), but appear absent in the genomes of others, including *Platyhelminthes* (planaria), *Hexapoda* (*Drosophila*), or *Craniata* (vertebrates). Consequently, transitive RNAi generating secondary sequences upstream of the region targeted by siRNAs was not observed in *Drosophila* or mouse (Schwarz et al., 2002; Roignant et al., 2003; Stein et al., 2003). Therefore, the absence of an RdRP gene in the genome can help as an indicator of the absence of the amplification loop.

Environmental and Systemic RNAi

It was shown in pioneering experiments in *C. elegans* that RNAi can be induced by simply soaking the worm into dsRNA solution (Tabara et al., 1998) or feed it bacteria expressing dsRNA (Timmons and Fire, 1998). These spectacular effects combined two distinct phenomena: (i) environmental RNAi where cells can uptake long dsRNA or small RNAs from the environment, and (ii) systemic RNAi where silencing can spread across cellular boundaries. While both phenomena can co-exist in one species, they might be distinct because the RNAi mediator spreading across cellular boundaries can be a different RNA molecule that the original inducing RNA molecule taken up from the environment. As the biology of systemic and environmental RNAi is complex and beyond the scope of this contribution, readers can look for more details into reviews on this topic, such as (Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010; Ivashuta et al., 2015).

dsRNA can be taken up *via* specific transmembrane channel mediated uptake (e.g., *C. elegans* or flower beetle) or through alternative endocytosis [e.g., in *Drosophila*, reviewed in more detail in (Whangbo and Hunter, 2008)]. Non-cell autonomous RNAi has been reported from parasitic nematodes (Geldhof et al., 2007), hydra (Chera et al., 2006), planaria (Newmark et al., 2003; Orii et al., 2003), some insects (Tomoyasu et al., 2008; Xu and Han, 2008), and plants (Himber et al., 2003). Some of the molecular mechanisms underlying systemic and environmental RNAi have been identified, such as dsRNA-transporting channels encoded by *sid-1* and *sid-2* genes (systemic RNAi-deficient), which function in systemic and environmental RNAi in *C. elegans* (Winston et al., 2002). *Sid-1* encodes a conserved transmembrane protein that forms a dsRNA channel and has homologs (but not necessarily orthologs) in a wide range of animals, including mammals (Feinberg and Hunter, 2003; Tomoyasu et al., 2008; Shih et al., 2009; Shih and Hunter, 2011; Cappelle et al., 2016). In contrast, *Sid-2*, which encodes a transmembrane protein, has only been found in several *Caenorhabditis* species (Winston et al., 2007; Dalzell et al., 2011).

In organisms displaying environmental and systemic RNAi, delivery of dsRNA could be used to intervene or harm. This phenomenon underlies strategies for crop protection [reviewed in more detail, for example, in (Cai et al., 2018)] and further discussed in the section *Horizontal Transfer of Small RNAs and RNAi Across Kingdoms*.

Notably, dsRNA itself has a potential to be used directly without producing a transgenic plant – as shown, for example, by topical application of dsRNA, which protected *Nicotiana benthamiana* and cowpea against infection with the potyvirus bean common mosaic virus (Worrall et al., 2019) and other cases [e.g., (Konakalla et al., 2019; Namgial et al., 2019)]. On a large scale, dsRNA feeding was used, for example, to protect bees against acute paralysis virus (Hunter et al., 2010), and spraying dsRNA solution was used to protect plants against fungus *Fusarium graminearum* (Koch et al., 2016).

OFF-TARGETING CONSIDERATIONS

One of the frequently raised questions is how specific and selective gene targeting by RNAi is. There is not a simple answer to that question, because there are several different strategies to induce RNAi and each of them has different potential for inducing off-targeting, i.e., downregulating an unintended target. Off-targeting was typically discussed as non-specific effects within one experimental model system [e.g., (Echeverri et al., 2006; Svoboda, 2007)]. In case of RNAi-mediated pest control, off-targeting would mainly consider effects on gene expression in other species than the targeted pest. There are two possible general effects on non-targeted species: (i) RNAi (typically siRNA-based) would induce miRNA-like repression of genes whose transcripts have complementarity to the seed sequence (wrong genes silenced in wrong species), (ii) RNAi would target gene(s) with high sequence similarity to dsRNA/siRNA (right gene (or its homologs) silenced in wrong species).

In addition, off-targeting in mammalian cells was also linked with a sequence-independent interferon response induced by long dsRNA. Although it is not clear whether environmental exposure to doses of dsRNA used for pest control would induce the interferon response in humans or other mammals, it is a testable and resolvable issue.

miRNA-Like Off-Targeting Effects in Other Species

miRNA-like off-target repression is a common off-targeting issue, particularly troubling RNAi experiments in mammalian cells, where it was shown that the off-target gene repression depends on the siRNA concentration and seed sequence (Jackson et al., 2003; Jackson et al., 2006). Several strategies have been proposed for achieving more selective RNAi in mammalian cells, including good experimental design (e.g., using the lowest effective siRNA concentration and employing specificity controls) or using RNAi-inducing agents with increased specificity—these include (i) chemical modifications eliminating activity of the "passenger"(non-targeting) siRNA strand or affecting seed pairing (Jackson et al., 2006; Chen et al., 2008;

Fluiter et al., 2009; Snead et al., 2013; Seok et al., 2016), and (ii) pools of more different siRNAs with the molarity of each seed sequence proportionally diluted. Accordingly, when considering this "miRNA-like" type of off-targeting, the two key factors are the mechanism of RNAi induction and the concentration of the RNAi-inducing molecule (leaving aside additional issues like small RNA sorting into different RNA silencing pathways and a varying crosstalk between miRNA and RNAi in different organisms). In general, a long dsRNA, which is converted into a siRNA pool or a pool of chosen siRNAs, principally represents a low if any risk of miRNA-like off-targeting in contrast to a single targeting siRNA (Stein et al., 2005; Hannus et al., 2014). However, exceptions may emerge: an RNAi screen with long dsRNA in *Drosophila* showed that some long dsRNA sequences yielded off-targeting, which stemmed from short tandem repeat sequences in the dsRNA (Ma et al., 2006).

Undesirable RNAi Effects in Non-Target Species

This issue is represented by targeting a homologous gene because of existing sequence similarity. This off-target effect is most likely to appear in closely related species in the environment treated with RNAi-based pest control. However, it is difficult to predict at which point the sequence divergence will render RNAi non-effective. As discussed above, a single nucleotide mismatch may be sufficient to prevent targeting, but this depends on the position and type of the mismatch (Du et al., 2005). Given the inhibitory effects of mismatches in the seed sequence and in the central part, 90% sequence identity with evenly distributed mismatches of an off-target homologous gene could be sufficiently diverged to lack perfect complementarity regions >17 nt. The effects on the off-target homolog would also depend on the concentration of RNAi-inducing agent; in one case in *C. elegans*, an 80% sequence identity of two genes yielded cross-interference, which was remedied by reducing concentration of microinjected dsRNA from 1 mg/ml to 100 μg/ml (Tabara et al., 1998). In a study of targeting a V-ATPase gene in the western corn rootworm gene with dsRNA, a silencing of its ortholog in the Colorado potato beetle (80% sequence identity) was observed but LC50 values showed a ten-fold difference in activity (Baum et al., 2007). Analysis of ten insect families in four different orders showed that the dsRNA targeting the *Snf7* gene in western corn rootworm was only active in a subset of species in the *Chrysomelidae* family (leaf beetles) whose *Snf7* genes had >90% identity with the dsRNA sequence (Bachman et al., 2013). While percentage of the sequence identity may be an arbitrary factor as the distribution of mismatches in the sequence is also important, these numbers imply that 80%–90% sequence identity is around threshold for functional RNAi.

An additional important factor is how large the off-target gene downregulation will manifest as a biologically relevant off-target phenotype. RNAi-mediated silencing of gene homologs in other species will likely be less efficient than downregulation of the desired target, because the same amount of dsRNA will produce less functional siRNAs in non-targeted species. Therefore, while off-targeting may be detectable by qPCR, it could be tolerated without adverse effects.

HORIZONTAL TRANSFER OF SMALL RNAS AND RNAi ACROSS KINGDOMS

In 2012, a study suggested that miRNAs from ingested plants could traverse into the mammalian bloodstream and suppress genes in the liver (Zhang et al., 2012). The report received a lot of attention and spurred a major debate because of implications these data could have. We reviewed this issue in detail in the aforementioned report (Paces et al., 2017), including three problematic areas that lacked strong experimental support: (i) the mechanism of transport from the digestive system through the bloodstream to the cells, (ii) the effector complex structure, particularly its loading with single-stranded methylated plant miRNA, (iii) the targeting stoichiometry consistent with the above-mentioned picomolar range of miRNA K_D. Some of the follow-up studies supported the existence of functionally relevant "xenomiRs" in humans and other mammals, while other studies questioned or rejected the idea (Paces et al., 2017). A recent survey of 824 datasets from human tissue and body fluids argues that human xenomiRs are likely artifacts (Kang et al., 2017). Among the strong arguments against biologically relevant dietary xenomiRs in humans were: the minimal fraction of xenomiRs (0.001% of host human miRNA counts), apparent batch effects of xenomiRs, no significant enrichment in sequencing data from tissues and body fluids exposed to dietary intake (e.g., liver), no significant depletion in tissues and body fluids that are relatively separated from the main bloodstream (e.g., brain and cerebro-spinal fluid), and, remarkably, the observation that the majority (81%) of body fluid xenomiRs would stem from rodents, an unlikely dietary source but common experimental material. These data argue that miRNAs from the diet are not uptaken by mammals and integrated into their miRNA pathways. At the same time, organisms with environmental and systemic RNAi can be susceptible to dietary uptake of dsRNA or small RNA. This was already shown in the pioneering RNAi experiments mentioned above – soaking in dsRNA or feeding dsRNA-expressing bacteria could suppress gene expression in *C. elegans* (Tabara et al., 1998; Timmons and Fire, 1998).

Consequently, trans-kingdom RNAi potential could be exploited in plants expressing dsRNA and selectively targeting RNAi-sensitive pests with an outcome of choice, e.g., repelling the pest, immobilizing it, sterilizing it (Bhatia et al., 2012), or killing it (Baum et al., 2007; Mao et al., 2007; Bhatia et al., 2012; Zhang et al., 2015; Kola et al., 2016). Processing of expressed dsRNA by plant's RNA silencing machinery, which could reduce amount of dsRNA ingested by a pest or cause off-targeting of plant genes, can be prevented by localizing dsRNA expression into chloroplasts (Zhang et al., 2015). Given the genome sequence diversity and relatively high sequence specificity of RNAi, an RNAi-based pesticide could represent a biodegradable, highly selective pesticide with an adjustable selectivity for the pest control [reviewed, for example, in (Kunte et al., 2020)].

Every new technology brings safety concerns. If the small RNAs can spread, could an RNAi-inducing transgene in a plant or topical application of dRNA/siRNA also affect a non-targeted

organisms? What could be the consequences? In principle, the off-targeting risk is inherent to the RNAi approach, but it can be monitored and significantly reduced by a proper experimental design. Furthermore, if RNAi were induced transiently (i.e., through dsRNA or siRNA), the transient nature of RNAi would allow recovery from the off-targeting within days in the species lacking an RdRP amplification loop producing secondary siRNAs. It could take longer if the off-targeting triggered transitive RNAi in the species with an RdRP and/or could induce transcriptional silencing. Transgenerational silencing [reviewed in (Rechavi and Lev, 2017)] has variable duration. In *C. elegans*, RNAi targeting genes expressed in the soma typically affects only the F1 progeny, although exceptional transgenerational silencing for up to 13 generations was also reported (Minkina and Hunter, 2017). Importantly, the probability of inducing a long transgenerational off-target effect in an organism other than the targeted one is negligible for dsRNA sequences with good sequence divergence from closely related species.

RESISTANCE TO RNAi

There is always a risk of resistance to RNAi. In the case of an RNAi-based pesticide, one could expect selection for mutations affecting RNAi efficiency rendering the RNAi-based pesticide ineffective. This could either involve accumulation of mutations within the sequence of the pest target gene (rather unlikely for long dsRNA), mutations within RNAi pathway factors of the pest (including uptake mechanisms), or evolution of *bona fide* RNAi suppressor proteins, which are known defense strategy against RNAi used by viruses (Roth et al., 2004; Haasnoot et al., 2007; Nayak et al., 2010).

Animals lacking RNAi may be viable and fertile, as shown in an *rde-1* mutant in *C. elegans* (Tabara et al., 1999). In fact, wild type isolates of *C. elegans* vary in the RNAi response and may exhibit different degrees of resistance to RNAi (Tijsterman et al., 2002; Elvin et al., 2011; Felix et al., 2011) despite the fact that some of the mutations could make them more susceptible to infection (Felix et al., 2011). A similar scenario could be expected for pests targeted by RNAi that would acquire some mutations in the RNAi pathway. Since most mutations would be recessive, the manifestation of resistance (and strong positive selection) would require homozygosity. Evolved resistance against dsRNA was reported in western corn rootworm (Khajuria et al., 2018). It was a single locus recessive mutation resulting in impaired luminal uptake of dsRNA (Khajuria et al., 2018). Therefore, one should consider the reproduction and life cycle of the targeted pest to develop an optimal treatment regimen to reduce (or not facilitate) the probability of occurrence of homozygous RNAi pathway mutants.

SUMMARY

RNAi offers selective gene targeting in a species-specific manner. RNAi induced by long dsRNA or unmodified siRNA offers a species-specific biodegradable pesticide. RNAi can be a particularly potent tool against pests that display environmental and systemic RNAi. The risk of potential off-targeting effects can be minimized when selecting the target and its sequence. Off-targeting effects can be monitored in closely related species and targets and, if identified, they would disappear after termination of the RNAi treatment.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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siRNA Specificity: RNAi Mechanisms and Strategies to Reduce Off-Target Effects

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Short interfering RNAs (siRNAs) are processed from long double-stranded RNA (dsRNA), and a guide strand is selected and incorporated into the RNA-induced silencing complex (RISC). Within RISC, a member of the Argonaute protein family directly binds the guide strand and the siRNA guides RISC to fully complementary sites on-target RNAs, which are then sequence-specifically cleaved by the Argonaute protein—a process commonly referred to as RNA interference (RNAi). In animals, endogenous microRNAs (miRNAs) function similarly but do not lead to direct cleavage of the target RNA but to translational inhibition followed by exonucleolytic decay. This is due to only partial complementarity between the miRNA and the target RNA. SiRNAs, however, can function as miRNAs, and partial complementarity can lead to miRNA-like off-target effects in RNAi applications. Since siRNAs are widely used not only for screening but also for therapeutics as well as crop protection purposes, such miRNA-like off-target effects need to be minimized. Strategies such as RNA modifications or pooling of siRNAs have been developed and are used to reduce off-target effects.

Keywords: siRNAs, microRNAs, off-target effects, RISC, RNAi

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INTRODUCTION

Double-stranded RNA (dsRNA) as trigger for RNA interference (RNAi) has been discovered decades ago in plants and nematodes (Baulcombe, 1996; Fire et al., 1998). Although these organisms are rather distant, the underlying mechanisms are remarkably conserved. dsRNA is generated by transcription or enzymes such as RNA-dependent RNA polymerases (RdRPs), which use single-stranded RNA as a template to generate long dsRNA (Meister and Tuschl, 2004; Mello and Conte, 2004; Sharp and Zamore, 2000). This RNA is further processed to short interfering RNAs (siRNAs), which serve as guides for the RNA-induced silencing complex (RISC) that binds and sequence-specifically cleaves complementary target RNAs (Zamore and Haley, 2005). This process is commonly referred to as RNAi. However, long dsRNA is toxic for animal organisms with more sophisticated immune systems that are capable of sensing long dsRNA as "foreign" as such RNAs could, for example, result from viral infections (Schlee and Hartmann, 2016). However, a breakthrough was reached when it was found that short siRNAs bypass immune sensing and can be used for gene knockdown also in higher organisms such as mammals (Elbashir et al., 2001). Besides broad usage in basic research, siRNAs have now been developed to target genes for therapy and indeed the first siRNAs reached the market (Dorsett and Tuschl, 2004; Sheridan, 2017). In addition to the therapeutic use in mammals, RNAi is also being explored as crop protection agent (Zhang et al., 2017). dsRNA directed against pests such as fungi, nematodes, or insects is sprayed

onto the leaves of plants and upon uptake selectively affects growth of distinct target species. Since dsRNA is species specific, is a natural product, and with no genetically modified organism needs to be generated, such strategies are considered highly promising next-generation plant protection agents. Nevertheless, both for human disease and for plant protection purposes, high specificity is critical and off-target effects need to be minimized (Seok et al., 2018). The following chapters will summarize principles of small RNA functions and highlight strategies to reduce off-target effects in gene knockdown experiments.

RNAi COMPONENTS IN ANIMALS AND PLANTS

Both in animals and plants, long dsRNA is processed to double-stranded siRNAs by Dicer-like enzymes (Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001). In *Arabidopsis thaliana*, four Dicer-like enzymes exist (referred to as DCL1-4), which are specialized for the generation of different classes of small RNAs (Bologna and Voinnet, 2014). DCL1 processes primary microRNAs (miRNA) to 21-nt-long mature miRNAs. DCL2 is involved in antiviral strategies and cleave viral dsRNA to 21/22-nt-long siRNAs, which target viral RNAs. DCL3 functions in silencing processes targeting transposable elements and produces siRNAs of about 24 nt in length. Finally, DCL4 generates 21 nt transacting siRNAs (tasiRNAs), which silence specific endogenous genes. Except for DCL1 that engages already-folded and dsRNA precursors, DCL2-4 cooperate with specialized RdRPs that generate the dsRNA substrates from single-stranded transcripts [for more information on plant Dicer enzymes, see Bologna and Voinnet (2014), Fukudome and Fukuhara (2017)].

In animals, Dicer enzymes are typically less diverse (Meister and Tuschl, 2004). Dicer enzymes belong to the RNase III enzyme family, which recognizes the ends of long dsRNAs and cleave the RNA about 21 nt from the end (Treiber et al., 2019). Dicer-like enzymes possess two catalytic RNase III domains, which cleave both strands and due to their positioning on the dsRNA, leave two nucleotides 3′ overhangs (Filipowicz, 2005; Treiber et al., 2019). In subsequent steps, commonly referred to as RISC loading, a member of the Argonaute protein family recognizes particularly the 3′ overhangs and selects one strand of the duplex to become the guide strand (also referred to as the antisense strand). The other strand, referred to as passenger strand, is degraded (Dueck and Meister, 2014; Ipsaro and Joshua-Tor, 2015; Sheu-Gruttadauria and MacRae, 2017). While structural information for most Ago proteins is lacking (including all plant Ago proteins), human Ago2 is reasonably well understood and is presented as an example for general structural features of Ago proteins. Argonaute proteins are structurally highly conserved and typically contain four domains (**Figures 1A,B**), as follows: the N domain, which has been implicated in siRNA duplex unwinding (Kwak and Tomari, 2012), the PAZ domain that anchors the 3′ end of the selected guide strand (Lingel et al., 2003; Ma et al., 2004; Yan et al., 2003), the MID domain that binds the 5′ end of the guide strand (Ma et al., 2005; Parker et al., 2004), and the PIWI domain. The PIWI domain has structural similarities to RNase H, which cleaves RNA molecules in RNA-DNA hybrids (Song et al., 2004; Wang et al., 2009; Yuan et al., 2005). Thus, some, but not all Argonaute proteins are endonucleases that cleave the target RNA in siRNA-target RNA hybrids using a catalytic tetrad in their active centers (Nakanishi et al., 2012) (**Figure 1C**). These proteins are referred to as Slicer enzymes (Liu et al., 2004; Meister et al., 2004). Furthermore, structural work revealed that MID domains of animal and plant Ago proteins display a sequence bias regarding the 5′ terminal nucleotide, which is important for sorting specific classes of small RNAs into their correct silencing pathways (Frank et al., 2010; Frank et al., 2012; Mi et al., 2008).

Plant Argonaute proteins are functionally diverse and are involved in various different gene silencing processes (Carbonell, 2017). In *A. thaliana*, Ago1, Ago7, and Ago10 bind to miRNAs and silence target genes. Ago4 has been implicated in RNA-directed DNA methylation and chromatin is epigenetically modified by this pathway. Generally, through their small RNA partner, plant Ago proteins are also involved in antiviral or bacterial defense mechanisms as well as responses to herbivore attack (Pradhan et al., 2017; Sibisi and Venter, 2020).

In plants as well as some animal species, which tolerate long dsRNA, the RNAi signal can be amplified by RdRPs (Maida et al., 2011). These enzymes use a siRNA strand bound to its target RNA as primer and synthesize the complementary strand to the target RNA resulting in a long dsRNA, which again enters Dicer processing and a second wave of siRNAs against a specific target is generated. Furthermore, in plants and also in some animal species, particularly nematodes such as *Caenorhabditis elegans*, siRNAs can be actively transported between cells and tissues [see, for example, (Das et al., 2019) for more information on this broad topic]. Remarkably, siRNA signals can also be inherited and target RNAs can be silenced over many generations. This process has been studied in *C. elegans* and is known as transgenerational gene silencing (TGS) [for more information on this exciting topic, please see, for example, (Iwasaki et al., 2015; Lev et al., 2019; Schraivogel and Meister, 2014)].

miRNA-GUIDED GENE SILENCING

MiRNAs are found in almost all plants and animals and in contrast to siRNAs, are transcribed from distinct miRNA genes (Bartel, 2009; Kim et al., 2009). RNA polymerase II transcription results in capped and poly-adenylated primary miRNA transcripts (pri-miRNAs), which are recognized and processed by the nuclear microprocessor containing the RNase III enzyme Drosha and its interaction partner DGCR8 (Cai et al., 2004; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003; Lee et al., 2004). Drosha/DGCR8 do not exist in plants and therefore DCL1 processes primary miRNA hairpins to mature miRNAs already in the nucleus of plant cells (Bologna and Voinnet, 2014; Bologna et al., 2018; Fukudome and Fukuhara, 2017).

Within pri-miRNAs, the miRNA strand itself is embedded in the stem of a local hairpin and the microprocessor cleaves the hairpin at the base of the stem. The resulting hairpin

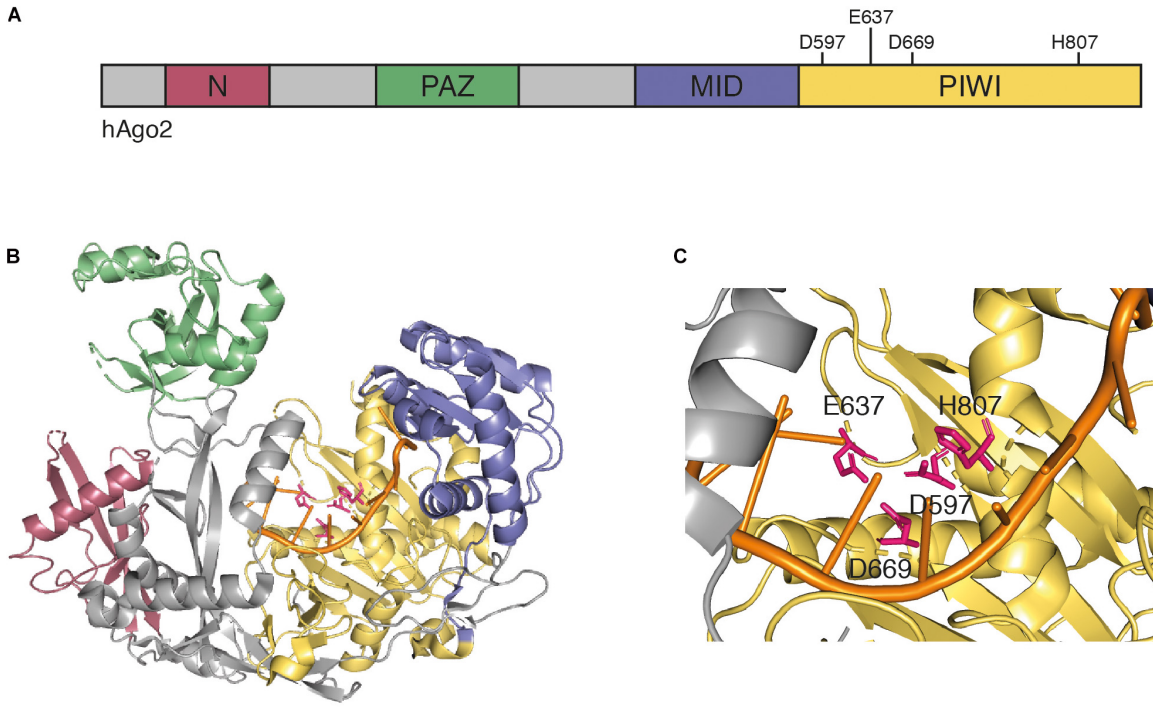

FIGURE 1 | (A) Schematic representation of human Ago2. Argonaute proteins contain four conserved domains: the N domain (red), the PAZ domain (green), the MID domain (blue), and the PIWI domain (yellow). The PIWI domain contains the four catalytic residues D597, E637, D669, and H807 that are required for mRNA target cleavage. **(B)** Crystal structure of human Ago2 loaded with a siRNA (PDB ID 5JS1) (Schirle et al., 2016). The four domains are colored as in panel **(A)**, the catalytic residues are highlighted in pink, and the 5′ region of the loaded siRNA is shown in orange. **(C)** Detailed view of catalytic center of the Ago2 structure from panel **(B)**.

structure, referred to as miRNA precursor (pre-miRNA), is exported to the cytoplasm by the export receptor Exportin-5 in animals (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, Dicer binds to the end of the hairpin and cleaves off an approx. 21-nt-long dsmiRNA intermediate, which is reminiscent of a siRNA duplex described above (Zhang et al., 2002). Consistently, RISC loading processes are similar to siRNAs and both in plants and in animals require the action of heat shock protein 90 (HSP90) that holds Ago proteins in a loading competent open conformation (Dueck and Meister, 2014; Iki et al., 2012; Iwasaki et al., 2010; Miyoshi et al., 2010). Moreover, miRNAs function like siRNAs in case the miRNA and the target RNA are fully or almost fully complementary (Doench et al., 2003). This mechanism is predominant in plants (Song et al., 2019). In animals, however, target RNA binding as well as the mechanism of gene silencing is different. MiRNA-target sites are typically located in the 3′ untranslated region (UTR) of mRNAs (Bartel, 2009). Nucleotides 2–8 of the miRNA represent the seed sequence, which is generally fully complementary to the target site while the remaining sequence is often only partially paired (Rajewsky and Socci, 2004). This incomplete pairing prevents Slicer-mediated cleavage as it is observed also in siRNA-guided knockdown studies. Instead, Argonaute proteins recruit a member of the GW protein family, which coordinates the following steps in miRNA-guided gene silencing (Behm-Ansmant et al., 2006; Jakymiw et al., 2005; Liu et al., 2005; Meister et al., 2005). GW proteins are characterized by glycine-tryptophane repeats and are referred to as TNRC6 proteins in mammals (Pfaff and Meister, 2013; Pfaff et al., 2013). GW proteins establish interactions with the poly(A) tail of the mRNAs as well as deadenylase complexes including the CCR4/NOT complex or PAN2/3 leading to translational repression, deadenylation of the mRNA, and, finally, to the removal of the 5′ cap by decapping enzymes (**Figure 2**). The unprotected mRNA is then degraded by 5′–3′ exoribonucleases [for more details, see Braun et al. (2013), Jonas and Izaurralde (2015), Krol et al. (2010)]. Translation repression without site-specific cleavage has also been observed in plants of target sites that are fully complementary but located in the 3′ UTR of mRNAs (Brodersen et al., 2008). Since GW proteins are not conserved in plants, the extent of this type of miRNA action remains to be further investigated in plants (Song et al., 2019).

MiRNA-LIKE OFF-TARGET EFFECTS IN RNAi STRATEGIES

Although miRNA-guided gene silencing is distinct from siRNA-guided knockdown experiments, the pathways are intertwined and miRNAs can function as siRNAs and vice versa. This is particularly important for off-target effects observed in RNAi experiments (Seok et al., 2018). SiRNAs may, in addition to their fully complementary on-targets, bind to an undefined number of miRNA-like target sites in 3′ UTRs of mRNAs using their

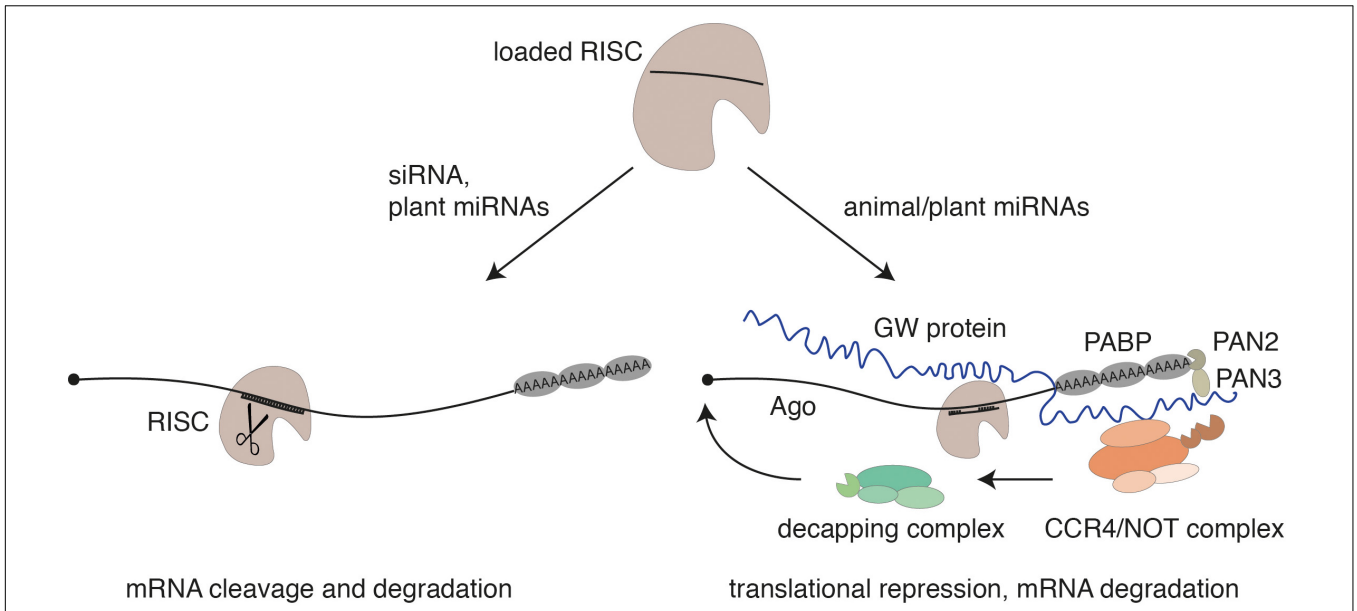

FIGURE 2 | The guide strand of the siRNA or a miRNA is loaded into an Argonaute protein. In case of a perfect complementarity through siRNAs or miRNAs (which is often observed in plants), a catalytically active Argonaute protein cleaves the mRNA as part of RISC (left). As miRNAs in animals are only partially complementary to their target RNAs, Slicer-facilitated cleavage is impaired. In this case, Argonaute recruits a member of the GW protein family. These proteins mediate the interaction with further downstream acting factors like poly-(A)-binding proteins (PABPs) or the deadenylase complexes PAN2/PAN3 and CCR4/NOT. This leads to translational repression, deadenylation, decapping, and 5′–3′ exonucleolytic decay of the mRNA. Translational repression by miRNAs has also been observed for plant miRNAs.

seed sequence. This will lead to silencing and unwanted off-target effects. Since such sequences are only 6–7 nt long, these unspecific target sites are hardly predictable and are thus very difficult to avoid. Indeed, miRNA-like off-target effects are highly problematic in large-scale RNAi screening approaches, and many hits are false positive and caused by off-target effects [e.g., (Birmingham et al., 2006; Buehler et al., 2012; Fedorov et al., 2006; Jackson et al., 2006b)]. Thus, strategies that control for or even reduce or eliminate such off-target effects are urgently needed.

In RNAi-mediated pest control, such off-target effects might not be predominantly problematic for the plant system since such a translational control system might be rather rare. However, in strategies, in which plants express si- or shRNAs that are taken up by animals and are toxic to defined species, off-target effects need to be considered. For example, non-target animals might incorporate these RNAs as well and, although perfect complementary target RNAs are absent, the expression of partially complementary sites could be affected through the endogenous miRNA system.

STRATEGIES TO REDUCE miRNA-LIKE OFF-TARGET EFFECTS

SiRNAs are typically designed to avoid complementary sequences to other RNAs besides the on-target. However, miRNA-like seed matches are difficult to predict because they statistically occur very frequently on mRNAs and not all such matches are always leading to significant knockdown effects. A conclusive strategy to monitor such effects are whole transcriptome sequencing in case target organisms and cells are identifiable. However, molecules with strongly reduced off-target effects would be the most desirable approach. To reduce miRNA-like off-target activities, two main strategies have been developed (Jackson and Linsley, 2010; Seok et al., 2018). First, siRNA guide strands are chemically modified within their seed region particularly at position 2 from the 5′ end (Jackson et al., 2006a). Such modifications are either 2′-*O*-methylations or locked nucleic acid incorporations, in which the 2′-OH is chemically linked to the 4′ carbon of the ribose (Elmen et al., 2005). Both modifications weaken the interaction between the guide strand and the target. Since seed matches are short, such interactions are much stronger affected by this mild destabilization than siRNAs, which are typically fully complementary to their on-target. Thus, miRNA-like off-target interactions are reduced while on-target silencing is not compromised. In addition to the modification at position 2, other modifications have also been explored [for more details, please see Seok et al. (2018)]. A second approach to reduce off-target effects is pooling of multiple siRNAs. It is important to notice that miRNA-like off-target effects are specific to individual sequences. Thus, reducing the concentration of the applied siRNAs will also reduce miRNA-like off-target effects. This could be achieved by administration of very low concentrations (Persengiev et al., 2004). However, this would also directly affect on-target activity. An elegant way of lowering concentrations of siRNAs is siRNA pooling. Individual siRNAs within such a pool are directed against the same on-target at different positions, but each individual siRNA has a unique off-target signature. Consequently, all siRNAs act synergistically

on the same on-target RNA. In complex pools, concentrations of individual siRNAs are very low and thus miRNA-like off-target effects are diluted out and cannot be measured anymore. Based on these ideas, three main pooling strategies are currently used. First, in the so-called smartPools, four individual siRNAs are combined. However, the complexity of such pools is low and thus the desired dilution effects are often not very pronounced. In contrast, endoribonuclease-produced siRNA pools (esiRNAs) are generated *in vitro* by recombinant RNase III digestion of long dsRNA (Kittler et al., 2005). These pools are then applied to cell cultures and, since these highly complex pools contain hundreds of different siRNAs, sequence-specific off-targets are not observed (Hannus et al., 2014; Kittler et al., 2005). A third strategy are so-called siPOOLs, which are highly complex but in contrast to esiRNAs, well defined. Up to 30 different siRNAs are designed and generated *in vitro* and such pools eliminate off-target effects even when a single siRNA with a pronounced off-target is included into the pool (Hannus et al., 2014).

Chemical modifications are the preferred choice when siRNAs are used for therapeutic purposes. For drug development, single and well-defined molecule species are preferred since broad toxicological validations are required during clinical trials and final approval. SiRNA pooling strategies are preferred in individual knockdown studies for research purposes or in genome-wide RNAi screening studies. Such pools are cost-efficient and thus genome-scale libraries are available.

CONCLUSION FOR RNA-BASED CROP PROTECTION AND OUTLOOK

Plants and animals with rather primitive immune systems tolerate long dsRNA and process it to siRNAs for gene silencing. One strategy in RNA-based crop protection is to spray dsRNA directed against pest-specific genes onto plants (Cai et al., 2018). Fungi or herbivores will take up these RNAs and process them to complex siRNA mixtures similar to esiRNA pools. This will kill or affect growth of the pathogens. Since such complex pools are naturally generated from dsRNAs in nematodes, insects, or fungi, miRNA-like off-target activity might be neglectable, when dsRNA is applied. In higher organisms such as mammals, the dsRNA will be fully degraded while transitioning through the digestive tract and only free nucleosides will be taken up. Thus, the administration of dsRNA to plants is an elegant and presumably very safe way of plant protection. SiRNAs are designed sequence specifically, and effects on other even highly related species could be minimized. Furthermore, since dsRNA is a natural product that is present in human diet, it might be better accepted by local communities than other plant protection strategies including the generation of genetically modified organisms (GMOs) or the use of conventional pesticides.

AUTHOR CONTRIBUTIONS

GM structured and wrote the text. JN wrote the text and designed figures. Both authors contributed to the article and approved the submitted version.

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Conflict of Interest: GM is a co-founder of siTOOLs biotech.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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